

Form PTO-1390 (REV 10-95) U S DEPARTMENT OF COMMERCE AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER GDV-001.01
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 CFR 1.5) To Be Assigned 10/019325
INTERNATIONAL APPLICATION NO. PCT/CA00/00774	INTERNATIONAL FILING DATE 29 June 2000	PRIORITY DATE CLAIMED 30 June 1999; 17 September 1999
TITLE OF INVENTION Heparin Compositions That Inhibit Clot Associated Coagulation Factors		
APPLICANT(S) FOR DO/EO/US Jeffrey I. Weitz, et al.		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
<p>1. <input checked="" type="checkbox"/> This is the FIRST submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration.</p> <p>4. <input type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)).</p> <ul style="list-style-type: none"> a. <input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> has been transmitted by the International Bureau c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US) <p><input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).</p> <p><input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <ul style="list-style-type: none"> a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> has been transmitted by the International Bureau c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made. <p><input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p><input checked="" type="checkbox"/> An UNEXECUTED oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p>		
Items 11. to 16. below concern document(s) or information included:		
<p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input checked="" type="checkbox"/> A FIRST preliminary amendment.</p> <p><input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</p> <p>14. <input type="checkbox"/> A substitute specification</p> <p>15. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>16. <input type="checkbox"/> Other items of information.</p>		

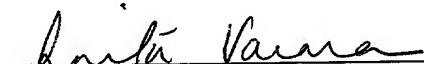
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Michael Phelan

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U.S. APPLICATION NO. (If known, 37 CFR 1.5)		INTERNATIONAL APPLICATION NO. PCT/CA00/00774	ATTORNEY DOCKET NUMBER GDV-001.01
16/019325			
17. <input checked="" type="checkbox"/> The following fees are submitted:		CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492(A)(1)-(5):			
Search Report has been prepared by the EPO or JPO.....\$890.00			
International preliminary examination fee paid to USPTO (37 CFR 1.482)\$710.00			
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)).....\$740.00			
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO\$1040.00			
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).\$100.00			
ENTER APPROPRIATE BASIC FEE AMOUNT =		\$890.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than months from the earliest claim priority date (37 CFR 1.492(e)).		<input type="checkbox"/> 20 <input type="checkbox"/> 30	\$
CLAIMS	NUMBER FIELD	NUMBER EXTRA	RATE
Total claims	37 - 20 =	17	X \$18.00 \$306.00
Independent claims	3 - 3 =	0	X \$84.00 \$
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)			+ \$280.00 \$
		TOTAL OF ABOVE CALCULATIONS =	\$1196.00
Reduction of ½ for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).		\$	
		SUBTOTAL =	\$
Processing fee of \$130.00 for furnishing the English translation later than months from the earliest claim priority date (37 CFR 1.492(f)).		<input type="checkbox"/> 20 <input type="checkbox"/> 30	\$
		+	\$
		TOTAL NATIONAL FEE =	\$
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property.		+	\$
		TOTAL FEES ENCLOSED =	\$1196.00
		Amount to be refunded:	\$
		Charged:	\$
<input checked="" type="checkbox"/> A check in the amount of <u>\$1,196.00</u> to cover the above fees is enclosed. <input type="checkbox"/> Please charge my Deposit Account No. <u>06-1448</u> in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>06-1448</u> . A duplicate copy of this sheet is enclosed			
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.			
SEND ALL CORRESPONDENCE TO:		 Anita Varma NAME 43,221 REGISTRATION NUMBER	
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10/019325

531 Rec'd PCT/PT 21 DEC 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Weitz, Jeffrey I., et al.	Atty. Docket No.:	<u>GDV - 001.01</u>
Serial No.:	Not yet assigned	Examiner:	Not yet assigned
Filing Date:	Not yet assigned	Group Art Unit:	Not yet assigned
Title:	<i>Heparin Compositions that Inhibit Clot Associated Coagulation Factors</i>		

Assistant Commissioner for Patents
Washington, DC 20231

Certificate of Mailing

I hereby certify that the instant "Preliminary Amendment" is being deposited with the U.S. Postal Service as Express Mail on the date set forth below in a postage-prepaid envelope addressed to:
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12/21/01

By: Michael Phelan

Date of Signature and Mail Deposit

Michael Phelan

Preliminary Amendment

Please amend the above-identified application as set forth below:

In the claims

Please cancel claims 3-34, without prejudice to the subject matter claimed therein. Applicants reserve the rights to prosecute the subject matter claimed therin in continuing and/or divisional applications.

Please add the following new claims:

--35. (new) A medium molecular weight heparin (MMWH) composition comprising a mixture of sulfated oligosaccharides having molecular weights ranging from about 6,000 Daltons to about 12,000 Daltons wherein at least 15% of said oligosaccharides have at least one pentasaccharide sequence.

36. (new) The MMWH composition of claim 35, wherein the oligosaccharides are of sufficient length to inhibit fibrin-bound thrombin and fluid-phase thrombin by catalyzing antithrombin, and to inhibit thrombin generation by catalyzing factor Xa inactivation by antithrombin.
37. (new) The MMWH composition of claim 35, wherein said oligosaccharides are of sufficient length to bridge antithrombin or heparin cofactor II (HCII) to thrombin but do not bridge thrombin to fibrin.
38. (new) The MMWH composition of claim 35, wherein at least 31% of said oligosaccharides have a molecular weight greater than or equal to about 7,800.
39. (new) The MMWH composition of claim 35, wherein said oligosaccharides have molecular weights ranging from about 8,000 Daltons to about 10,000 Daltons.
40. (new) The MMWH composition of claim 38, wherein said oligosaccharides have molecular weights of about 8,500 Daltons.
41. (new) The MMWH composition of claim 35, wherein at least 20% of said oligosaccharides have at least one pentasaccharide sequence.
42. (new) The MMWH composition of claim 41, wherein at least 30% of said oligosaccharides have at least one pentasaccharide sequence.
43. (new) The MMWH composition of claim 42, wherein at least 35% of said oligosaccharides have at least one pentasaccharide sequence.

44. (new) The MMWH composition of claim 43, wherein at least 40% of said oligosaccharides have at least one pentasaccharide sequence.

45. (new) The MMWH composition of claim 35, wherein said MMWH composition has an anti-factor IIa activity of about 40 U/mg to about 100 U/mg, and an anti-factor Xa activity of about 90 U/mg to about 150 U/mg.

46. (new) The MMWH composition of claim 45, wherein said MMWH composition has an anti-factor IIa activity of about 60 U/mg to about 75 U/mg, and an anti-factor Xa activity of about 100 U/mg to about 125 U/mg.

47. (new) The MMWH composition of claim 35, wherein said oligosaccharides have a polydispersity of 1.1 to 1.5.

48. (new) The MMWH composition of claim 47, wherein said oligosaccharides have a polydispersity of 1.2 to 1.4.

49. (new) The MMWH composition of claim 35, wherein said MMWH composition has anti-factor Xa activity and anti-factor IIa activity and wherein the ratio of anti-factor Xa activity to anti-factor IIa activity is from about 2:1 to about 1:1.

50. (new) The MMWH composition of claim 35 comprising a mixture of oligosaccharides derived from heparin having antithrombin and HCII related anticoagulant activity; having sufficient length to bridge antithrombin or HCII to thrombin, but do not bridge thrombin to fibrin; having at least 20%, 25%, 30%, 35%, or 40% oligosaccharides with at least one or more pentasaccharide sequence; and being enriched for oligosaccharides having a molecular weight range from about 8,000 to 10,000 Daltons.

51. (new) The MMWH composition of claim 35 comprising a mixture of oligosaccharides derived from heparin having sufficient length to bridge antithrombin or HCII to thrombin, but do not bridge thrombin to fibrin; having at least 20%, 25%, 30%, 35%, or 40% oligosaccharides with at least one or more pentasaccharide sequence; having a mean molecular weight of about 8,000 to 9,800 Daltons; and having a polydispersity of about 1.1 to about 1.5.
52. (new) The MMWH composition of claim 35 comprising a mixture of oligosaccharides derived from heparin having sufficient length to bridge antithrombin or HCII to thrombin, but do not bridge thrombin to fibrin; having at least 20%, 25%, 30%, 35%, or 40% oligosaccharides with at least one or more pentasaccharide sequence; being enriched for oligosaccharides having a molecular weight range from about 8,000 to 10,000 Daltons; having a mean molecular weight of about 8,000 to 9,800 Daltons; and having similar anti-factor Xa and anti-factor IIa activities wherein the ratio of anti-factor Xa activity to anti-factor IIa activity is from about 2:1 to about 1:1.
53. (new) The MMWH composition of claim 50 comprising a mixture of oligosaccharides derived from heparin having a polydispersity of about 1.1 to about 1.5.
54. (new) A MMWH composition comprising a mixture of oligosaccharides derived from heparin having sufficient length to bridge antithrombin or HCII to thrombin, but do not bridge thrombin to fibrin; having a mean molecular weight of about 8,000 to 9,800 Daltons; having a polydispersity of about 1.1 to about 1.5; having an anti-factor Xa activity from about 80 IU/mg to

about 105 IU/mg; and having an anti-factor IIa activity from about 20 IU/mg to about 150 IU/mg.

55. (new) The MMWH composition of claim 54 comprising a mixture of oligosaccharides derived from heparin wherein at least 30%, 35%, 40%, 45%, or 50% of said oligosaccharides have a molecular weight greater than or equal to 8,000 Daltons.
56. (new) The MMWH composition of claim 53 comprising a mixture of oligosaccharides derived from heparin wherein at least 30%, 35%, 40%, 45%, or 50% of said oligosaccharides have a molecular weight greater than or equal to 8,000 Daltons; having similar anti-factor Xa and anti-factor IIa activities wherein the ratio of anti-factor Xa activity to anti-factor IIa activity is from about 2:1 to about 1:1; having an anti-factor Xa activity from about 80 IU/mg to about 105 IU/mg; and having an anti-factor IIa activity from about 20 IU/mg to about 150 IU/mg.
57. (new) A method of treating a thrombotic condition in a subject comprising administering to said subject a pharmacologically acceptable dose of the MMWH composition of claim 35.
58. (new) The method of claim 57, wherein said thrombotic condition is arterial thrombosis, coronary artery thrombosis, venous thrombosis, or pulmonary embolism.
59. (new) The method of claim 57, wherein said MMWH composition is administered by injection.
60. (new) A method of preventing the formation of a thrombus in a subject at risk of developing thrombosis comprising administering to said subject a

pharmacologically acceptable dose of the MMWH composition of claim 35.

61. (new) The method of claim 60, wherein said subject is at increased risk of developing thrombosis due to a medical condition which disrupts hemostasis.
62. (new) The method of claim 61, wherein the medical condition is coroanary artery disease, or atherosclerosis.
63. (new) The method of claim 60, wherein said subject is at increased risk of developing thrombosis due to a medical procedure.
64. (new) The method of claim 63, wherein the medical procedure is cardiac surgery, cardipulmonary bypass, catheterization, or atherectomy.
65. (new) The method of claim 64, wherein the catheterization is cardiac catherterization.
66. (new) A method of inhibiting thrombus formation in a patient comprising the step of administering to the patient a pharmacologically acceptable dose of the MMWH composition of claim 35.
67. (new) A composition comprising the MMWH composition of claim 35 and a pharmaceutically acceptable carrier.
68. (new) A method of treating deep vein thrombosis in a patient comprising administering to said patient undergoing orthopedic surgery a therapeutically effective amount of the MMWH composition of claim 35.

69. (new) A method of preventing a pulmonary embolism in a subject comprising administering to said subject a therapeutically effective amount of the MMWH composition of claim 35.

Remarks

For the Examiner's convenience, Applicants provide the following table which points to exemplary support for the newly added claims in the specification as originally filed:

<i>Claim Number</i>	<i>Support</i>
Claim 35	Pages 2, 4
Claim 36	Page 1
Claim 37	Page 4
Claims 38-40, 45, 46	Page 3
Claims 41-44, 47-49	Page 4
Claims 50-56	Pages 3-5
Claim 57	Page 5
Claim 58, 66	Pages 5-6
Claims 59-65	Page 6
Claim 67	Page 19
Claim 68	Page 48
Claim 69	Pages 48-49

Therefore, no new matter has been added. Entry of this amendment is respectfully requested.

CONCLUSION

If there are any other fees due in connection with the filing of this Preliminary Amendment not accounted for in the accompanying check, please charge the fees to our **Deposit Account No. 06-1448**. If a fee is required for an extension of time under 37 C.F.R. § 1.136 not accounted for above, such an extension is requested and the fees should also be charged to our Deposit Account.

Respectfully submitted,
Foley Hoag

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TITLE: HEPARIN COMPOSITIONS THAT INHIBIT CLOT ASSOCIATED COAGULATION**FACTORS****FIELD OF THE INVENTION**

This invention relates generally to compositions and methods for the treatment of cardiovascular disease. More particularly, the present invention relates to modifying thrombus formation and growth by administering a medium molecular weight heparin (MMWH) composition that, *inter alia*, is capable of (1) inactivating fluid-phase thrombin as well as thrombin which is bound either to fibrin in a clot or to some other surface by catalyzing antithrombin; and (2) inhibiting thrombin generation by catalyzing factor Xa inactivation by antithrombin III (ATIII). In addition, the present invention provides methods and compositions useful for treating cardiovascular disease.

BACKGROUND OF THE INVENTION

Heparin acts as an anticoagulant by binding to antithrombin and markedly increasing the rate at which it inhibits activated factor X (factor Xa) and thrombin. The interaction of heparin with antithrombin is mediated by a unique pentasaccharide sequence that is randomly distributed on about one-third of the heparin chains. To catalyze thrombin inhibition by antithrombin, heparin must bind simultaneously to the enzyme and the inhibitor. Provision of this bridging function requires pentasaccharide-containing heparin chains with a minimum molecular weight of 5,400 Daltons. Even heparin chains of this minimum size may be of insufficient length to bridge thrombin to antithrombin if the pentasaccharide is located in the middle of the heparin chain rather than at either end. In contrast, longer pentasaccharide-containing heparin chains are able to provide this bridging function regardless of the location of the pentasaccharide within the heparin chain.

Like heparin, low molecular weight heparin (LMWH) also acts as an anticoagulant by activating antithrombin. However, with a mean molecular weight of about 4,500 to 5,000 Daltons, the majority of the LMWH chains are too short to bridge thrombin to antithrombin. Consequently, the inhibitory activity of LMWH against thrombin is considerably less than that of heparin.

Although heparin is an efficient inhibitor of fluid-phase thrombin, it is limited in its ability to inactivate thrombin bound to fibrin, e.g., clot-bound thrombin. The resistance of fibrin-bound thrombin to inactivation by the heparin-antithrombin complex reflects the fact that heparin bridges thrombin to fibrin to form a ternary fibrin-thrombin-heparin complex. Formation of this ternary complex heightens the affinity of thrombin for fibrin 20-fold (from a Kd of 3 μ M to an apparent Kd of 150 nM). By occupying the heparin-binding site on thrombin, the heparin chain that tethers thrombin to fibrin prevents heparin within the heparin-antithrombin complex from bridging antithrombin to the fibrin-bound thrombin. This explains why fibrin-bound thrombin is protected from inactivation by the heparin-antithrombin complex.

Moreover, with a mean molecular weight of 4,500 to 5,000 Daltons, the majority of the chains of LMWH are also too short to bridge thrombin to fibrin. However, because most of the LMWH chains also are too short to bridge thrombin to antithrombin, LMWH is a poor inhibitor of both fluid-phase and fibrin-bound thrombin.

In view of the foregoing, there still remains a need in the art for improved heparin compositions that are useful, for example, for inhibiting thrombogenesis associated with cardiovascular disease. An ideal heparin composition would be one which can pacify the clot by inactivating fibrin-bound thrombin and by

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blocking thrombin generation, thereby preventing the reactivation of coagulation that occurs once treatment is stopped. More particularly, an ideal heparin composition would be one in which the heparin chains are too short to bridge thrombin to fibrin, but are of a sufficient length to bridge antithrombin to thrombin. The present invention fulfills these and other needs.

5 **SUMMARY OF THE INVENTION**

The present invention provides Medium Molecular Weight Heparin (MMWH) compositions comprising heparin chains that are too short to bridge thrombin to fibrin, but that are of a sufficient length to bridge antithrombin to thrombin. Bridging of thrombin to fibrin is only effected by heparin chains that are larger than 12,000 Daltons. Thus, the minimum molecular weight of heparin needed to provide this bridging
10 function is considerably greater than that needed to bridge antithrombin to thrombin. As such, the MMWH compositions of the present invention were designed to fit within this window. With a molecular weight range of about 6,000 to about 12,000 Daltons, the MMWH compositions of the present invention are comprised of heparin chains or sulfated oligosaccharides that are too short to bridge thrombin to fibrin. However, a lower limit of 6,000 Daltons was specifically chosen to ensure that all of the heparin chains of the MMWH compositions are of a sufficient length to bridge antithrombin to thrombin regardless of where the pentasaccharide sequence is located within the heparin chains. For these reasons, the MMWH compositions of the present invention, unlike heparin, inhibit fibrin-bound thrombin and fluid-phase thrombin equally well.

The MMWH compositions of the present invention can pacify the thrombus (or, interchangeably, clot) by inactivating fibrin-bound thrombin, thereby preventing reactivation of coagulation once treatment is stopped, and can block thrombin generation by inhibiting factor Xa. As such, the present invention provides methods of using the MMWH compositions to treat cardiovascular diseases. As explained above, the MMWH compositions of the present invention are a mixture of sulfated oligosaccharides typically having molecular weights ranging from about 6,000 Daltons to about 12,000 Daltons and, even more preferably, from about 8,000 Daltons to about 10,000 Daltons. In a preferred embodiment, the MMWH compounds of the present invention have a mean molecular weight of about 9,000 Daltons. In one embodiment, at least 31% of the MMWH compositions have a molecular weight greater than or equal to 7,800 Daltons. In another embodiment, at least 25% of the MMWH compositions have a molecular weight greater than or equal to 10,000 Daltons. Such MMWH compositions can readily be prepared from standard or
30 unfractionated heparin.

Moreover, the MMWH compositions of the present invention typically have similar anti-factor Xa and anti-factor IIa activities. In a preferred embodiment, the ratio of anti-factor Xa activity to anti-factor IIa activity ranges from about 2:1 to about 1:1 and, more preferably, from about 1.5:1 to about 1:1. In contrast, LMWHs, for example, have significantly more anti-factor Xa activity than anti-factor IIa activity. In a preferred embodiment, the anti-factor Xa activity of the MMWH compositions of the present invention ranges from about 80 U/mg to about 155 U/mg, preferably 90 U/mg to about 150 U/mg and, more preferably, from about 100 U/mg to about 125 U/mg. In an even more preferred embodiment, the MMWH compositions of the present invention have an anti-factor Xa activity of about 115 U/mg. In a preferred embodiment, the anti-factor IIa activity of the MMWH compositions of the present invention ranges from
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about 20 U/mg to about 150 U/mg, preferably 40 U/mg to about 100 U/mg and, more preferably, from about 60 U/mg to about 75 U/mg. In an even more preferred embodiment, the MMWH compositions of the present invention have an anti-factor IIa activity of about 65 U/mg.

As described above, the MMWH compositions of the present invention comprise heparin chains that are too short to bridge thrombin to fibrin, but are of a sufficient length to bridge antithrombin to thrombin. Consequently, unlike heparin, the MMWH compositions of the present invention inactivate both fibrin-bound thrombin and free thrombin. Moreover, although most low molecular weight heparin (LMWH) chains are of insufficient length to bridge thrombin to fibrin, they are also too short to bridge antithrombin to thrombin. Consequently, the MMWH compositions of the present invention are considerably better than LMWH at inactivating fibrin-bound thrombin. In addition, although hirudin can inactivate fibrin-bound thrombin, it has no effect on thrombin generation because it is a selective inhibitor of thrombin. Consequently, in contrast to hirudin, the MMWH compositions of the present invention inhibit thrombin generation by catalyzing factor Xa inactivation by antithrombin. Thus, by blocking thrombin generation as well as by inhibiting fibrin-bound thrombin, the MMWH compositions of the present invention overcome the limitations of heparin, LMWH and hirudin, particularly in the setting of acute arterial thrombosis.

Selected MMWH compositions of the invention are also contemplated that are enriched for oligosaccharides having an optimal molecular weight range providing particularly advantageous properties as illustrated herein. These MMWH compositions comprise a mixture of oligosaccharides derived from heparin characterized by one, two, three, four, five, or six ,or more of the following characteristics:

- (a) having antithrombin- and heparin cofactor II (HCII)-related anticoagulant activity *in vitro*;
- (b) the oligosaccharides are too short to bridge thrombin to fibrin, but are of a sufficient length to bridge antithrombin or HCII to thrombin;
- (c) having at least 15%, 20%, 25%, 30%, 35%, or 40% oligosaccharides with at least one or more pentasaccharide sequence;
- (d) enriched for oligosaccharides having a molecular weight range from about 6,000 to about 11,000; 7,000 to 10,000; 7,500 to 10,000; 7,800 to 10,000; 7,800 to 9,800; or 7,800 to 9,600; 8,000 to 9,600;
- (e) the oligosaccharides have a mean molecular weight of about 7,800 to 10,000, preferably 7,800 to 9,800, more preferably 8,000 to 9,800;
- (f) at least 30%, 35%, 40%, 45%, or 50% of the oligosaccharides have a molecular weight greater than or equal to 6000 Daltons, preferably greater than or equal to 8000 Daltons;
- (g) a polydispersity of 1.1 to 1.5, preferably 1.2 to 1.4, most preferably 1.3;
- (h) having similar anti-factor Xa and anti-factor IIa activities, preferably a ratio of anti-factor Xa activity to anti-factor IIa activity from about 2:1 to about 1:1 and, more preferably, from about 1.5:1 to about 1:1;
- (i) an anti-factor Xa activity from about 80 IU/mg to about 155 IU/mg, preferably 90 IU/mg to about 130 IU/mg, more preferably, from about 95 IU/mg to about 120 IU/mg and, most preferably 100-110 IU/mg;

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- (j) an anti-factor IIa activity from about 20 IU/mg to about 150 IU/mg; preferably 40 IU/mg to about 100 IU/mg, more preferably, from about 80 IU/mg to about 100 IU/mg, most preferably about 90-100 IU/mg.

In accordance with an aspect of the invention a selected MMWH composition of the invention has
5 the characteristics (a), (b), (c) and (d); (a) (b), (c), and (e); (b), (c), (e), and (g); (b), (d), (c), (e), and (h); (b)
(c), (d), and (g); (b), (e), (g), (i), and (j); (b), (e), (f), (g), (i) and (j); or (a) through (j).

“Enriched for oligosaccharides” refers to a MMWH composition comprising at least 50%, 55%,
60%, 65%, 70%, 75%, or 80% oligosaccharides within a specified or restricted molecular weight range (e.g.
6,000 to 11,000; 7,000 to 10,000; 7,800 to 10,000; 7,800 to 9,800; or 8,000 to 9,600).

10 As a result of their ability to (1) inhibit fibrin-bound thrombin as well as fluid-phase thrombin by catalyzing antithrombin, and (2) inhibit thrombin generation by catalyzing factor Xa inactivation by antithrombin, the MMWH compositions of the present invention can be used to treat cardiovascular diseases, including unstable angina, acute myocardial infarction (heart attack), cerebral vascular accidents (stroke), pulmonary embolism, deep vein thrombosis, arterial thrombosis, etc. As such, the present invention provides methods and pharmaceutical compositions for treating such cardiovascular diseases.

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In one embodiment, the present invention provides a method of treating a thrombotic condition in a subject, the method comprising administering to the subject a pharmacologically acceptable dose of a MMWH composition of the invention. The composition may comprise a mixture of sulfated oligosaccharides having molecular weights ranging from about 6,000 Daltons to about 12,000 Daltons and, even more preferably, of about 8,000 Daltons to about 10,000 Daltons. In a preferred embodiment, the MMWH composition has a mean molecular weight of about 9,000 Daltons. In another preferred embodiment, the MMWH composition is a selected MMWH composition having an optimal molecular weight range as described herein. In preferred aspects of this embodiment, the thrombotic condition includes, but is not limited to, venous thrombosis (e.g., deep-vein thrombosis), arterial thrombosis and coronary artery thrombosis. In this embodiment, the MMWH composition inhibits thrombus formation and growth, for example, by inhibiting fibrin-bound thrombin and fluid-phase thrombin, and by inhibiting thrombin generation by catalyzing factor Xa inactivation by antithrombin. Preferably, administration of the compounds is achieved by parenteral administration (e.g., by intravenous, subcutaneous and intramuscular injection).

30 In another embodiment, the present invention provides a method of preventing the formation of a thrombus in a subject at risk of developing thrombosis, the method comprising administering to the subject a pharmacologically acceptable dose of a MMWH composition of the invention. The composition may comprise a mixture of sulfated oligosaccharides having molecular weights ranging from about 6,000 Daltons to about 12,000 Daltons and, even more preferably, of about 8,000 Daltons to about 10,000 Daltons. In a
35 preferred embodiment, the MMWH composition has a mean molecular weight of about 9,000 Daltons. In another embodiment, the MMWH composition is a selected MMWH composition having an optimal molecular weight range as described herein. In one aspect of this embodiment, the subject is at increased risk of developing a thrombus due to a medical condition which disrupts hemostasis (e.g., coronary artery disease, atherosclerosis, etc.). In another aspect of this embodiment, the subject is at increased risk of

developing a thrombus due to a medical procedure (e.g., cardiac surgery (e.g., cardiopulmonary bypass), catheterization (e.g., cardiac catheterization, percutaneous transluminal coronary angioplasty), atherectomy, placement of a prosthetic device (e.g., cardiovascular valve, vascular graft, stent, etc.). In this embodiment, the MMWH compositions can be administered before, during or after the medical procedure. Moreover, administration of the MMWH compositions is preferably achieved by parenteral administration (e.g., by intravenous, subcutaneous and intramuscular injection).

The invention also contemplates the use of a MMWH composition of the invention in the preparation of a medicament for treating a thrombotic condition, or preventing the formation of a thrombus in a subject at risk of developing thrombosis; use of a MMWH composition of the invention in the preparation of a medicament for inhibiting fibrin-bound thrombin and thrombin generation in a subject; use of a MMWH composition of the invention in the preparation of a medicament for treating deep vein thrombosis; and use of a MMWH composition of the invention in the preparation of a medicament for preventing pulmonary embolism in a subject.

Other features, objects and advantages of the invention and its preferred embodiments will become apparent from the detailed description which follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B illustrate the effects of varying heparin concentrations on thrombin (IIa) binding to fibrin (A) and on thrombin's apparent affinity for fibrin (B).

Figure 2 illustrates the percentage of α -thrombin (α -IIa), γ -thrombin (γ -IIa) or RA-thrombin (RA) that binds to fibrin monomer-sepharose in the absence or presence of heparin.

Figure 3 illustrates the effect of hirugen (Hg), prothrombin fragment 2 (F2) or antibody against exosite 2 (Wab) on thrombin (IIa) binding to fibrin monomer-sepharose in the absence or presence of 250 nM heparin.

Figure 4 illustrates the ternary fibrin-thrombin-heparin complex wherein thrombin (IIa) binds to fibrin (Fn) via exosite 1 and heparin (Hp) binds to both Fn and exosite 2 on IIa.

Figure 5 illustrates the effect of fibrin monomer (Fm) on the rates of thrombin inhibition by antithrombin (■) or heparin cofactor II (●) in the presence of 100 nM heparin. Each point represents the mean of at least 2 separate experiments, while the bars represent the SD.

Figures 6A and 6B illustrate the inhibitory effects of 4 μ M fibrin monomer () on the rates of thrombin inhibition by antithrombin (A) or heparin cofactor II (B) in the absence or presence of heparin at the concentrations indicated. Each point represents the mean of at least 2 experiments, while the bars represent the SD.

Figure 7 illustrates the interaction of γ -thrombin (γ -IIa), Quick 1 dysthrombin (Q1-IIa) or RA-IIa with fibrin (Fn) in the presence of heparin (Hp). Non-productive ternary complexes are formed because γ -IIa and Q1-IIa have an altered exosite 1, whereas RA-IIa has reduced affinity for Hp.

Figure 8 illustrates the effect of binary or ternary complex formation on the K_m for hydrolysis of N-p-Tosyl-Gly-Pro-Arg-p-nitroanilide by α -thrombin (α -IIa), γ -thrombin (γ -IIa), or RA-thrombin (RA-IIa). Binary complexes include thrombin-fibrin (IIa-Fn), and thrombin-heparin (IIa-Hp), whereas the ternary

complex is thrombin-fibrin-heparin (IIa-Fn-Hp). Each bar represents the mean of at least two experiments, while the lines represent the SD.

Figure 9 illustrates the effect of unfractionated heparin (UFH) and a 6,000 Da heparin fraction (MMWH) on thrombin (IIa) binding to fibrin.

5 Figure 10 illustrates the inhibitory effects of 4 μ M fibrin monomer on the rate of thrombin inhibition by antithrombin (AT) or heparin cofactor II (HCII) in the presence of heparin or a MMWH composition of the present invention. Each bar represents the mean of at least 2 separate experiments, while the lines represent the SD.

10 Figure 11 illustrates the cumulative patency in % of standard heparin (SH), low molecular weight heparin (LMWH), a MMWH composition of the present invention, and hirudin (HIR) in the prevention model study.

15 Figure 12 illustrates the effect of standard heparin (SH), low molecular weight heparin (LMWH), a MMWH composition of the present invention, and hirudin (HIR) on cumulative blood loss at 30 minutes.

20 Figures 13A and 13B illustrate the efficacy of LMWH and a MMWH composition of the present invention, in the arterial thrombosis model (A), and the effect of LMWH and a MMWH composition of the present invention on blood loss (B).

25 Figure 14 shows comparative effects of a MMWH composition of the present invention and LMWH on APTT.

30 Figure 15 shows comparative effects of LMWH and a MMWH composition of the present invention on the anti-Xa level.

Figure 16 is a schematic diagram of the procedure.

35 Figure 17 shows a modified Wessler model Clot Weight by percentage following treatment with a MMWH composition of the present invention.

Figure 18 shows a comparison of LMWH and a MMWH composition of the present invention: Prophylaxis model.

Figure 19 shows a comparison of LMWH and a MMWH composition of the present invention: Prophylaxis model.

Figure 20 shows a modified Wessler model of clot radioactivity by percentage following treatment with a MMWH composition of the present invention.

30 Figure 21 is a comparison of LMWH and a MMWH composition of the present invention: prophylaxis model.

Figure 22 is a comparison of LMWH and a MMWH composition of the present invention: prophylaxis model.

35 Figure 23 is a comparison of LMWH and a MMWH composition of the present invention in a treatment model.

Figure 24 is a comparison of LMWH and a MMWH composition of the present invention in a treatment model.

Figure 25 shows a comparison of LMWH and a MMWH composition of the present invention on thrombus accretion.

Figure 26 shows a comparison of LMWH and a MMWH composition of the present invention on thrombus accretion.

Figure 27 shows treatment of DVT in chronic rabbit model clot accretion with a MMWH composition of the present invention.

5 Figure 28 shows treatment of DVT in chronic rabbit model % change in clot weight with a MMWH composition of the present invention.

Figure 29 is a graph showing rates of AT inhibition of thrombin with heparinase-derived medium molecular weight (MMW) heparins \pm 4 μ M fibrin monomer.

10 Figure 30 is a graph showing rates of AT inhibition of thrombin with nitrous acid-derived medium molecular weight (MMW) heparins \pm 4 μ M fibrin monomer.

Figure 31 is a graph showing rates of AT inhibition of thrombin with periodate-derived medium molecular weight (MMW) heparins \pm 4 μ M fibrin monomer.

Figure 32 is a graph showing fold inhibition by fibrin monomer of the rate of thrombin inhibition by AT with heparinase and nitrous acid-derived MMW heparins.

15 Figure 33 is a graph showing fold inhibition by fibrin monomer of the rate of thrombin inhibition by AT with periodate-derived MMW heparins

Figure 34 is a graph showing rates of AT inhibition of Factor Xa with heparinase-derived medium molecular weight heparins.

20 Figure 35 is a graph showing rates of AT inhibition of Factor Xa with nitrous acid-derived medium molecular weight heparins.

Figure 36 is a graph showing rates of AT inhibition of Factor Xa with periodate-derived medium molecular weight heparins.

Figure 37 is a graph showing the effect of UFH and heparinase-derived medium molecular weight heparins on thrombin binding to fibrin clots.

25 Figure 38 is a graph showing the effect of UFH and nitrous acid-derived medium molecular weight heparins on thrombin binding to fibrin clots.

Figure 39 is a graph showing the effect of UFH and periodate-derived medium molecular weight heparins on thrombin binding to fibrin clots.

30 Figure 40 is a graph showing the effect of UFH and size restricted heparinase-derived medium molecular weight heparins on thrombin binding to fibrin clots.

Figure 41 is a graph showing the effect of UFH and size restricted nitrous acid-derived medium molecular weight heparins on thrombin binding to fibrin clots.

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

The present invention provides Medium Molecular Weight Heparin (MMWH) compounds that (1) 35 inhibit fibrin-bound thrombin as well as fluid-phase thrombin by catalyzing antithrombin, and (2) inhibit thrombin generation by catalyzing factor Xa inactivation by antithrombin. These MMWH compositions are a mixture of sulfated oligosaccharides having molecular weights ranging from about 6,000 Daltons to about 12,000 Daltons and, even more preferably, from about 8,000 Daltons to about 10,000 Daltons. In an embodiment, the MMWH compositions of the present invention have a mean molecular weight of about

9,000 Daltons. In one embodiment, at least 31% of the MMWH compositions have a molecular weight greater than or equal to 7,800 Daltons. In another embodiment, at least 25% of the MMWH compositions have a molecular weight greater than or equal to 10,000 Daltons.

More particularly, the MMWH compositions of the present invention can pacify the intense prothrombotic activity of the thrombus. The prothrombotic activity of the thrombus reflects the activity of fibrin-bound thrombin and platelet-bound activated factor X (factor Xa), both of which are relatively resistant to inactivation by heparin and LMWH. This explains why these agents are of limited efficacy in the setting of arterial thrombosis and why rebound activation of coagulation occurs when treatment is stopped. Moreover, although hirudin can, in contrast to heparin, inactivate fibrin-bound thrombin, it fails to block thrombin generation triggered by platelet-bound factor Xa. The ability of hirudin to inactivate fibrin-bound thrombin explains why direct thrombin inhibitors are superior to heparin for the short-term management of arterial thrombosis. However, any beneficial effects of these agents are rapidly lost once treatment is stopped because they fail to block thrombin generation that is triggered by platelet-bound factor Xa.

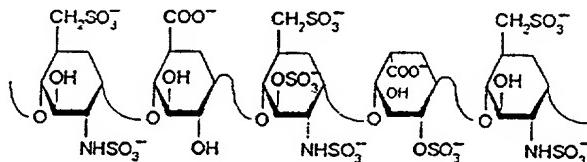
It has now been determined that fibrin-bound thrombin is resistant to inactivation by heparin because the heparin bridges thrombin to fibrin by binding to both fibrin and the heparin-binding site on thrombin with high affinity; the K_d for both the heparin-fibrin and the heparin-thrombin interaction is about 150 nM. Thrombin within this ternary fibrin-thrombin-heparin complex undergoes a conformational change at its active site that likely limits its reactivity with antithrombin. Furthermore, by occupying the heparin-binding site on thrombin, the heparin chain that tethers thrombin to fibrin prevents heparin within the heparin-antithrombin complex from bridging antithrombin to the fibrin-bound thrombin. This explains why thrombin within the ternary fibrin-thrombin-heparin complex is protected from inactivation by heparin or by LMWH chains that are of sufficient length to bridge thrombin to antithrombin. It is likely that a major contributing factor to both the resistance of acute arterial thrombi to these anticoagulants and rebound activation of coagulation after stopping treatment is the inability of heparin, LMWH or hirudin to pacify the intense prothrombotic activity of the thrombus.

In contrast to heparin, LMWH and hirudin, the MMWH compositions of the present invention can pacify the prothrombotic activity of the thrombus by inactivating fibrin-bound thrombin and by inhibiting thrombin generation by catalyzing factor Xa inactivation by antithrombin. More particularly, it has been discovered that the heparin chains of the MMWH compositions of the present invention are too short to bridge thrombin to fibrin, but are of sufficient length to bridge antithrombin to thrombin. Consequently, unlike heparin, the MMWH compositions of the present invention inactivate both fibrin-bound thrombin and free thrombin. Moreover, although most LMWH chains are of insufficient length to bridge thrombin to fibrin, they are also too short to bridge antithrombin to thrombin. Consequently, the MMWH compositions of the present invention are considerably better than LMWH at inactivating fibrin-bound thrombin. In addition, although hirudin can inactivate fibrin-bound thrombin, it has no effect on thrombin generation because it is a selective inhibitor of thrombin. Consequently, in contrast to hirudin, the MMWH compositions of the present invention inhibit thrombin generation by catalyzing factor Xa inactivation by antithrombin. Thus, by blocking thrombin generation as well as by inhibiting fibrin-bound thrombin, the

MMWH compositions of the present invention overcome the limitations of heparin, LMWH and hirudin, particularly in the setting of acute arterial thrombosis.

The MMWH compositions of the present invention typically have similar anti-factor IIa and anti-factor Xa activities. In a presently preferred embodiment, the ratio of anti-factor Xa activity to anti-factor IIa activity ranges from about 2:1 to about 1:1 and, more preferably, from about 1.5:1 to about 1:1. In contrast, LMWHs, for example, have significantly more anti-factor Xa activity than anti-factor IIa activity. In a preferred embodiment, the anti-factor Xa activity of the MMWH compositions of the present invention ranges from about 90 U/mg to about 150 U/mg and, more preferably, from about 100 U/mg to about 125 U/mg. In an even more preferred embodiment, the MMWH compositions of the present invention have an anti-factor Xa activity of about 115 U/mg. In a presently preferred embodiment, the anti-factor IIa activity of the MMWH compositions of the present invention ranges from about 40 U/mg to about 100 U/mg and, more preferably, from about 60 U/mg to about 75 U/mg. In an even more preferred embodiment, the MMWH compositions of the present invention have an anti-factor IIa activity of about 65 U/mg.

Selected MMWH compositions of the invention are also contemplated that are enriched for oligosaccharides having an optimal molecular weight range providing particularly advantageous properties as illustrated herein. These MMWH compositions comprise a mixture of oligosaccharides derived from heparin characterized by having antithrombin- and heparin cofactor II (HCII)-related anticoagulant activity *in vitro*. The compositions comprise heparin chains that are too short to bridge thrombin to fibrin, but are of a sufficient length to bridge antithrombin or HCII to thrombin. In particular, the compositions have at least 15%, 20%, 25%, 30%, 35%, or 40% heparin oligosaccharide chains with at least one or more pentasaccharide sequence. "Pentasaccharide sequence" refers to a key structural unit of heparin that consists of three D-glucosamine and two uronic acid residues (See the structure below). The central D-glucosamine residue contains a unique 3-O-sulfate moiety.



The pentasaccharide sequence represents the minimum structure of heparin that has high affinity for antithrombin (Choay, J. et al., Biochem Biophys Res Comm 1983; 116: 492-499). The binding of heparin to antithrombin through the pentasaccharide sequence results in a conformational change in the reactive center loop which converts antithrombin from a slow to a very rapid inhibitor. Consequently, a selected MMWH composition of the invention will be capable of inhibiting fibrin-bound thrombin as well as fluid-phase thrombin by catalyzing antithrombin, and inhibiting thrombin generation by catalyzing factor Xa inactivation by antithrombin. Preferably, the selected MMWH compositions of the invention are those that inhibit fibrin-bound thrombin and fluid-phase thrombin equally well.

The selected MMWH compositions comprise oligosaccharides having a molecular weight range from about 6,000 to about 11,000. In accordance with one aspect of the invention a MMWH composition is provided that is enriched for oligosaccharides having a molecular weight range of 7,800 to 8,800, preferably

- 10 -

7,800 to 8,600, more preferably 7,800 to 8,500, most preferably 8,000 to 8,500. In another aspect of the invention a MMWH composition is provided that is enriched for oligosaccharides having a molecular weight range of 9,000 to 10,000, preferably 9,200 to 9,800, more preferably 9,300 to 9,600, most preferably 9,400 to 9,600.

5 In an embodiment the invention also contemplates a MMWH composition of the invention comprising oligosaccharides with a mean molecular weight of 7,800 to 8,800, preferably 7,800 to 8,600, more preferably 7,800 to 8,500, most preferably 8,000 to 8,500. In another embodiment, the invention contemplates a MMWH composition of the invention comprising oligosaccharides with a mean molecular weight of 9,000 to 10,000, preferably 9,200 to 9,800, more preferably 9,300 to 9,600, most preferably 9,400 to 9,600.

10 A selected MMWH composition may have a polydispersity of 1.1 to 1.5, preferably 1.2 to 1.4, most preferably 1.3.

15 A selected MMWH composition of the invention may have similar anti-factor Xa and anti-factor IIa activities. In an embodiment, the ratio of anti-factor Xa activity to anti-factor IIa activity ranges from about 2:1 to about 1:1 and, more preferably, from about 1.5:1 to about 1:1. In a preferred embodiment, the anti-factor Xa activity ranges from about 80 IU/mg to about 155 IU/mg, preferably 90 IU/mg to about 130 IU/mg, more preferably, from about 95 IU/mg to about 120 IU/mg and, most preferably 100-110 IU/mg. In a preferred embodiment, the anti-factor IIa activity ranges from about 20 IU/mg to about 150 IU/mg; more preferably 40 IU/mg to about 100 IU/mg, and most preferably, from about 80 IU/mg to about 100 IU/mg. In an even more preferred embodiment, the compositions have an anti-factor IIa activity of about 90-100 IU/mg.

20 The MMWH compositions of the present invention can be prepared from low standard or unfractionated heparin or, alternatively, from low molecular weight heparin (LMWH).

25 In one embodiment, the MMWH compositions of the present invention can be obtained from unfractionated heparin by first depolymerizing the unfractionated heparin to yield lower molecular weight heparin and then isolating or separating out the MMWH fraction of interest. Unfractionated heparin is a mixture of polysaccharide chains composed of repeating disaccharides made up of a uronic acid residue (D-glucuronic acid or L-iduronic acid) and a D-glucosamine acid residue. Many of these disaccharides are sulfated on the uronic acid residues and/or the glucosamine residue. Generally, unfractionated heparin has an average molecular weight ranging from about 6,000 Daltons to 40,000 Daltons, depending on the source of the heparin and the methods used to isolate it. The unfractionated heparin used in the process of the present invention can be either a commercial heparin preparation of pharmaceutical quality or a crude heparin preparation, such as is obtained upon extracting active heparin from mammalian tissues or organs. The commercial product (USP heparin) is available from several sources (e.g., SIGMA Chemical Co., St. Louis, Missouri), generally as an alkali metal or alkaline earth salt (most commonly as sodium heparin). Alternatively, the unfractionated heparin can be extracted from mammalian tissues or organs, particularly from intestinal mucosa or lung from, for example, beef, porcine and sheep, using a variety of methods known to those skilled in the art (see, e.g., Coyne, Erwin, Chemistry and Biology of Heparin, (Lundblad,

R.L., *et al.* (Eds.), pp. 9-17, Elsevier/North-Holland, New York (1981)). In a presently preferred embodiment, the unfractionated heparin is porcine intestinal heparin.

Numerous processes for the depolymerization of heparin are known and have been extensively reported in both the scientific and patent literature, and are applicable to the present invention. Such processes are generally based on either chemical or enzymatic reactions. For instance, a lower molecular weight heparin can be prepared from standard, unfractionated heparin by benzylation followed by alkaline depolymerization; nitrous acid depolymerization; enzymatic depolymerization with heparinase; peroxidative depolymerization, etc. Generally methods are chosen that provide compositions with characteristics of a MMWH composition of the invention, in particular a composition of the invention with an optimal molecular weight range. Desired characteristics of a composition of the invention i.e. molecular weight range, mean or average molecular weight, polydispersity, anti-factor Xa activity, anti-factor IIa activity, etc, may be confirmed using standard methods (e.g. see the Examples herein). In a preferred embodiment, a composition of the invention is prepared from unfractionated heparin using nitrous acid depolymerization or heparinase depolymerization.

The unfractionated heparin may be depolymerized by contacting unfractionated heparin, under controlled conditions, to the actions of a chemical agent, more particularly, nitrous acid. The nitrous acid can be added to the heparin directly or, alternatively, it can be formed *in situ*. To generate the nitrous acid *in situ*, controlled amounts of an acid are added to a derivative of nitrous acid. Suitable acids include those which advantageously contain biologically acceptable anions, such as acetic acid and, more preferably, hydrochloric acid. Suitable derivatives of nitrous acid include a salt, an ether-salt or, more preferably, an alkali or alkaline-earth salt. In a presently preferred embodiment, a salt of nitrous acid, a water-soluble salt, more preferably, an alkali salt, such as sodium nitrite (NaNO_2), is used.

The depolymerization of unfractionated heparin is preferably carried out in a physiologically acceptable medium, thereby eliminating the problems associated with the use of a solvent that can be detrimental to the contemplated biological applications. Such physiologically acceptable media include, but are not limited to, water and water/alcohol mixtures. In a presently preferred embodiment, water constitutes the preferred reaction medium. In carrying out the depolymerization reaction, it is desirable to use stoichiometric amounts of the reagents (e.g., nitrous acid). The use of stoichiometric amounts of nitrous acid will ensure that when the desired degree of depolymerization is reached, the nitrous acid is entirely consumed. Typically, the weight ratio of unfractionated heparin to sodium nitrite (NaNO_2) ranges from about 100 to 2-4 and, more preferably, from about 100 to 3. Using a stoichiometric amount of nitrous acid avoids the need to "quench" a kinetic (ongoing) reaction with, for example, ammonium sulfamate and, in turn, prevents the formation of mixed salts (e.g., sodium and ammonium) of the lower molecular weight heparin intermediates.

In addition, other parameters, such as temperature and pH, are adjusted with respect to one another in order to obtain the desired products under the most satisfactory experimental conditions. For instance, the depolymerization reaction can be carried out at temperatures ranging from about 0° to 30°C. In fact, temperatures lower than 10°C can be used for the production of the desired products. However, in a preferred embodiment, the depolymerization reaction is carried out at ambient temperature, i.e., between

about 20°C and 28°C. Moreover, in a preferred embodiment, the depolymerization reaction is initiated and terminated by first lowering and then raising the pH of the reaction mixture. To initiate the depolymerization reaction, the pH of the reaction mixture is lowered to a pH of about 2.5 to 3.5 and, more preferably, to a pH of about 3.0. Similarly, to terminate the depolymerization reaction, the pH of the reaction mixture is raised to a pH of about 6.0 to 7.0 and, more preferably, to a pH of about 6.75. It should be noted that the progress of the reaction can be monitored by checking for the presence or absence of nitrous ions in the reaction mixture using, for example, starch-iodine paper. The absence of nitrous ions in the reaction mixture indicates that the reaction has gone to completion. The time required for the reaction to reach completion will vary depending on the reactants and reaction conditions employed. Typically, however, the reaction will reach completion in anywhere from about 1 hour to about 3 hours.

Once the reaction has reached completion, the MMWH compositions can be recovered using a number of different techniques known to and used by those of skill in the art. In one embodiment, the MMWH compositions are recovered from the reaction mixture by precipitation, ultrafiltration or chromatography methods. If the desired product is obtained by precipitation, this is generally done using, for example, an alcohol (*e.g.*, absolute ethanol). In a presently preferred embodiment, the MMWH composition is recovered from the reaction mixture using ultrafiltration methods. Ultrafiltration membranes of various molecular weight cuts-offs can advantageously be used to both desalt and define the molecular weight characteristics of the resulting MMWH compositions. Ultrafiltration systems suitable for use in accordance with the present invention are known to and used by those of skill in the art. The commercially available Millipore Pellicon ultrafiltration device is an exemplary ultrafiltration system that can be used in accordance with the present invention. This device can be equipped with various molecular weight cut-off membranes. In a presently preferred embodiment, the resulting MMWH composition is dialyzed or ultrafiltered against purified water (*i.e.*, distilled water (dH₂O)) using a Millipore Pellicon ultrafiltration device equipped with 6,000 Dalton molecular weight cut-off membranes.

After ultrafiltration, the retentate is then lyophilized, *i.e.*, freeze-dried, to give the MMWH composition. The molecular weight characteristics of the resulting MMWH composition can be determined using standard techniques known to and used by those of skill in the art. Such techniques include, for example, GPC-HPLC, viscosity measurements, light scattering, chemical or physical-chemical determination of functional groups created during the depolymerization process, *etc.* In a preferred embodiment, the molecular weight characteristics of the resulting MMWH composition are determined by high performance size exclusion chromatography in conjunction with multiangle laser light scattering (HPSEC-MALLS). Typically, the resulting MMWH composition has an average or mean molecular weight average (Mw) of about 9,000 Daltons. In a selected composition of the invention, the average or mean molecular weight is about 7,800 to 10,000 Daltons, preferably 7,800 to 9,800 Daltons.

Those of skill in the art will readily appreciate that the resulting MMWH compositions can be subjected to further purification procedures. Such procedures include, but are not limited to, gel permeation chromatography, ultrafiltration, hydrophobic interaction chromatography, affinity chromatography, ion exchange chromatography, *etc.* Moreover, the molecular weight characteristics of the MMWH compositions of the present invention can be determined using standard techniques known to and used by those of skill in

the art as described above. As explained, in a preferred embodiment, the molecular weight characteristics of the MMWH compositions of the present invention are determined by high performance size exclusion chromatography in conjunction with multiangle laser light scattering (HPSEC-MALLS).

MMWH compositions of the invention may be prepared by enzymatic depolymerization of heparin by heparinase (see for example, U.S. 3,766,167, and U.S. 4,396,762). In accordance with one aspect of the invention, a composition of the invention, particularly a selected composition with an optimal molecular weight range or restricted molecular weight range is prepared by a controlled heparinase depolymerization as described in EP0244236 (Nielsen and Ostergard; No. 87303836.8 published 04.11.87). Using this method a MMWH composition of the invention may be prepared with a desired weight average molecular weight by depolymerizing with heparinase to the corresponding number average molecular weight. The method measures the increase in light absorption (preferably at 230-235 nm i.e. ΔA_{235}) during the course of depolymerization, and depolymerization is stopped when the light absorption has reached a calculated value corresponding to the desired number average molecular weight and the corresponding desired weight average molecular weight.

In another embodiment, the MMWH compositions of the present invention may be obtained by a limited periodate oxidation/hydrolysis of heparin to yield a lower molecular weight heparin, and then isolating or separating out the MMWH fraction of interest. In the first step of this method, heparin is contacted with a limited amount of sodium periodate. In a presently preferred embodiment, the concentration of sodium periodate ranges from about 1 mM to about 50 mM and, more preferably, from about 5 mM to 20 mM. The pH of this reaction mixture ranges from about 3 to 11 and, more preferably, from about 6.5 to about 7.5. The limited periodate oxidation is generally carried out for about 18 hours. In the second step of this method, an alkaline hydrolysis is carried out after the periodate oxidation using metal alkalines, such as NaOH. In a preferred embodiment, the concentration of the metal alkaline, e.g., NaOH, ranges from about 0.1 N to about 1 N and, more preferably, is about 0.25 N. This step is carried out at a temperature ranging from about 0°C to about 50°C and, more preferably, at a temperature of about 25°C, for a time period of about 1 hour to about 10 hours and, more preferably, 3 hours. The desired MMWH compositions are obtained using known methods, such as gel-filtration, ion-exchange chromatography, ultrafiltration, dialysis, quaternary ammonium precipitation, and organic solvent precipitation, as described above. Moreover, the MMWH compositions can be further purified using the methods described above.

Using a limited periodate oxidation/hydrolysis method, a MMWH composition is prepared that is structurally distinct from known LMWH compounds. As described above, in one embodiment, the MMWH compositions of the present invention are prepared by a brief treatment of unfractionated heparin with periodate to yield a product that is oxidized at some of the sulfated uronic acid residues. These oxidized sites may be readily cleaved with base. Consequently, cleavage of the MMWH composition may not be random as is typically the case with the methods currently used to prepare LMWH. Moreover, the 2-O sulfated uronic acid residues that are susceptible to oxidation by periodate are located with some frequency proximal to pentasaccharide sequences. Consequently, the limited periodate/hydrolysis method of the present invention may result in lower molecular weight heparin chains that have the pentasaccharide sequence

located at the end of the chain which may leave the remainder of the heparin chain long enough to bridge to thrombin.

The MMWH compositions of the present invention are capable of, *inter alia*, (1) inhibiting fibrin-bound thrombin as well as fluid-phase thrombin by catalyzing antithrombin, and (2) inhibiting thrombin generation by catalyzing factor Xa inactivation by antithrombin. As such, the MMWH compositions of the present invention can be used to treat a number of important cardiovascular complications, including unstable angina, acute myocardial infarction (heart attack), cerebral vascular accidents (stroke), pulmonary embolism, deep vein thrombosis, arterial thrombosis, etc. In a preferred embodiment, the MMWH compositions of the present invention are used to treat arterial thrombosis. As such, in another embodiment, 10 the MMWH compositions of the present invention can be incorporated as components in pharmaceutical compositions that are useful for treating such cardiovascular conditions. The pharmaceutical compositions of the present invention are useful either alone or in conjunction with conventional thrombolytic treatments, such as the administration of tissue plasminogen activator (tPA), streptokinase, and the like, with conventional anti-platelet treatments, such as the administration of ticlopidine, and the like, as well as with intravascular intervention, such as angioplasty, atherectomy, and the like.

The MMWH compositions of this invention can be incorporated into a variety of formulations for therapeutic administration. More particularly, the MMWH compositions of the present invention can be formulated into pharmaceutical compositions by combination with appropriate, pharmaceutically acceptable carriers or diluents, and may be formulated into various preparations, preferably in liquid forms, such as slurries, solutions and injections. Administration of the MMWH compositions of the present invention is preferably achieved by parenteral administration (*e.g.*, by intravenous, subcutaneous and intramuscular injection). Moreover, the compounds can be administered in a local rather than systemic manner, for example via injection of the compounds directly into a subcutaneous site, often in a depot or sustained release formulation.

Suitable formulations for use in the present invention are found in Remington's Pharmaceutical Sciences (Mack Publishing Company, Philadelphia, PA, 17th Ed. (1985)), the teachings of which are incorporated herein by reference. Moreover, for a brief review of methods for drug delivery, see, Langer, *Science* 249:1527-1533 (1990), the teachings of which are incorporated herein by reference. The pharmaceutical compositions described herein can be manufactured in a manner that is known to those of skill in the art, *i.e.*, by means of conventional mixing, dissolving, levigating, emulsifying, entrapping or lyophilizing processes. The following methods and excipients are merely exemplary and are in no way limiting.

The MMWH compositions of the present invention are preferably formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Generally, pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be

prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents that increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

More particularly, for injection, the MMWH compositions can be formulated into preparations by dissolving, suspending or emulsifying them in an aqueous or nonaqueous solvent, such as vegetable or other similar oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol; and if desired, with conventional additives, such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives. Preferably, the compositions of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers, such as Hanks's solution, Ringer's solution, or physiological saline buffer.

In addition to the formulations described previously, the MMWH compositions can also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in a therapeutically effective amount. By a "therapeutically effective amount" or, interchangeably, "pharmacologically acceptable dose" or, interchangeably, "anticoagulantly effective amount," it is meant that a sufficient amount of the compound, i.e., the MMWH composition, will be present in order to achieve a desired result, e.g., inhibition of thrombus accretion when treating a thrombus-related cardiovascular condition, such as those described above by, for example, inactivating clot-bound thrombin, inhibiting thrombin generation by catalyzing factor Xa inactivation by antithrombin, etc. The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician. Determination of an effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

A treatment or composition of the invention may be administered to subjects that are animals, including mammals, and particularly humans. Animals also include domestic animals, including horses, cows, sheep, pigs, cats, dogs, and zoo animals.

Typically, the active product, i.e., the MMWH compositions, will be present in the pharmaceutical composition at a concentration ranging from about 2 µg per dose to 200 µg per dose and, more preferably, at a concentration ranging from about 5 µg per dose to 50 µg per dose. Daily dosages can vary widely, depending on the specific activity of the particular MMWH, but will usually be present at a concentration ranging from about 0.5 µg per kg of body weight per day to about 15 µg per kg of body weight per day and.

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more preferably, at a concentration ranging from about 1 µg per kg of body weight per day to about 5 µg per kg of body weight per day.

In addition to being useful in pharmaceutical compositions for the treatment of the cardiovascular conditions described above, one of skill in the art will readily appreciate that the active products, i.e., the
5 MMWH compositions, can be used as reagents for elucidating the mechanism of blood coagulation in vitro.

The invention will be described in greater detail by way of specific examples. The following examples are offered for illustrative purposes, and are not intended to limit the invention in any manner. Those of skill in the art will readily recognize a variety of noncritical parameters which can be changed or modified to yield essentially the same results.

10 EXAMPLES

Example 1

Experimental Findings

1.1 Clinical Limitations of Currently Available Anticoagulants:

40 Heparin, LMWH and direct thrombin inhibitors have limitations in acute coronary syndromes. In patients with unstable angina, there is a clustering of recurrent ischemic events after treatment with these agents is stopped (Theroux, P., et al. (1992) *N. Engl. J. Med.* 327:141-145; Granger, C.B., et al. (1996) *Circulation* 93:870-888; Oldgren, J., et al. (1996) *Circulation* 94 (suppl 1):I-431). This is due to reactivation of coagulation because there is an associated elevation in plasma levels of prothrombin fragments F1.2 (F1.2) and fibrinopeptide A (FPA), reflecting increased thrombin generation and thrombin activity, respectively (Granger, C.B., et al. (1995) *Circulation* 91:1929-1935). In patients with acute myocardial infarction, thrombolytic therapy with tissue plasminogen activator (t-PA) or streptokinase induces a procoagulant state characterized by elevated levels of FPA (Eisenberg, P.R., et al. (1987) *J. Am. Coll. Cardiol.* 10:527-529; Owen, J., et al. (1988) *Blood* 72:616-620), which are only partially reduced by heparin (Galvani, J., et al. (1994) *J. Am. Coll. Cardiol.* 24:1445-1452; Merlini, P.A., et al. (1995) *J. Am. Coll. Cardiol.* 25:203-209). This explains why adjunctive heparin does not reduce the incidence of recurrent ischemic events in patients receiving streptokinase (Collins, R., et al. (1996) *BMJ* 313:652-659), and is of only questionable benefit in those given t-PA (Collins, R., et al. (1996) *BMJ* 313:652-659). Although hirudin is better than heparin both as an adjunct to thrombolytic therapy and in patients with non-Q wave infarction who do not receive thrombolytic agents, the early benefits of hirudin are lost within 30 days
30 (GUSTO Investigators (1996) *N. Engl. J. Med.* 335(11):775-782). These findings suggest that there is a persistent thrombogenic stimulus that is resistant to both heparin and hirudin.

Similar results are seen in the setting of coronary angioplasty. Recurrent ischemic events occur in 6-8% of patients despite aspirin and high-dose heparin (Popma, J.J., et al. (1995) *Chest* 108:486-501). Although hirudin is superior to heparin for the first 72 hours after successful coronary angioplasty, its
35 benefits are lost by 30 days (Serruys, P.W., et al. (1995) *N. Engl. J. Med.* 333:757-763). Similarly, at 7 days, hirulog, a semi-synthetic hirudin analogue (Bitll, J.A., et al. (1995) *J. Med.* 333:764-769), is better than heparin at preventing recurrent ischemic events in patients undergoing angioplasty for unstable angina after acute myocardial infarction; by 30 days, however, there is no difference between hirulog and heparin (Bitll, J.A., et al. (1995) *J. Med.* 333:764-769). It is likely that both the resistance of acute arterial thrombi

to heparin, LMWH and hirudin and the reactivation of coagulation that occurs when treatment is stopped reflect the inability of these anticoagulants to pacify the intense prothrombotic activity of the thrombus.

1.2 Factors Responsible for the Prothrombotic Activity of Acute Arterial Thrombi:

Arterial thrombosis is triggered by vascular injury. Spontaneous or traumatic rupture of 5 atherosclerotic plaque exposes tissue factor which complexes factor VII/VIIa. The factor VIIa/tissue factor complex then initiates coagulation by activating factors IX and X. Although factor VIIa within the factor VIIa/tissue factor complex is rapidly inactivated by tissue factor pathway inhibitor (Broze GJ Jr. (1995) *Thromb. Haemost.* 74:90-93), arterial thrombi remain thrombogenic.

Studies in vitro have attributed the procoagulant activity of arterial thrombi to (a) thrombin bound 10 to fibrin (Hogg, P.J., et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:3619-3623; Weitz, J.I., et al. (1990) *J. Clin. Invest.* 86:385-391), or (b) factor Xa (and possibly factor IXa) bound to platelets within the thrombi (Eisenberg, P.R., et al. (1993) *J. Clin. Invest.* 91:1877-1883). Fibrin-bound thrombin can locally activate platelets (Kumar, R., et al. (1995) *Thromb. Haemost.* 74(3):962-968) and accelerate coagulation (Kumar, R., et al. (1994) *Thromb. Haemost.* 72:713-721), thereby inducing an intense procoagulant state. By triggering thrombin generation, platelet-bound factor Xa (and IXa) augments this procoagulant state.

Both fibrin-bound thrombin and platelet-bound factor Xa are resistant to inactivation by heparin and LMWH (Hogg, P.J., et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:3619-3623; Weitz, J.I., et al. (1990) *J. Clin. Invest.* 86:385-391; Teitel, J.M., et al. (1983) *J. Clin. Invest.* 71:1383-1391; Pieters, J., et al. (1988) *J. Biol. Chem.* 263:15313-15318), thereby explaining their inability to pacify the procoagulant activity of acute arterial thrombi. Hirudin can inactivate fibrin-bound thrombin (Weitz, J.I., et al. (1990) *J. Clin. Invest.* 86:385-391), but fails to block thrombin generation triggered by platelet-bound clotting factors. In support of this concept, hirudin reduces the levels of FPA, but has no effect on F1.2 levels in patients with unstable 20 angina (Granger, C.B., et al. (1995) *Circulation* 91:1929-1935).

There is mounting evidence that both fibrin-bound thrombin and platelet-bound factor Xa contribute to the intense procoagulant activity of thrombi. Thus, the ability of a washed plasma clot to accelerate coagulation when incubated in unanticoagulated whole blood cannot be blocked by either hirudin or tick anticoagulant peptide (TAP), a direct inhibitor of factor Xa that unlike heparin and LMWH inactivates platelet-bound factor Xa as well as free factor Xa (Waxman, L., et al. (1990) *Science* 248:593-596). In contrast, a combination of hirudin and TAP abolishes the procoagulant activity of plasma clots. 30 suggesting that pacification of acute arterial thrombi requires agents that not only inhibit fibrin-bound thrombin, but also block thrombin generation triggered by platelet-bound factor Xa. Development of these agents requires an understanding of the mechanisms by which fibrin-bound IIa and platelet-bound factor Xa are protected from inactivation by heparin, LMWH and hirudin.

1.3 Mechanisms by Which Fibrin-bound Thrombin is Protected from Inactivation by Heparin:

Studies indicate that thrombin binding to fibrin is more complex in the presence of heparin than in its absence, and the consequence of thrombin/fibrin interactions has now been better delineated.

1.3.1 Thrombin/Fibrin Interactions in the Absence of Heparin:

In the absence of heparin, α -thrombin binds to fibrin with a $K_d = 2 \mu\text{M}$. Binding is mediated by exosite 1, the substrate-binding site on thrombin (Fenton, J.W. II, et al. (1988) *Biochemistry* 27:7106-7112)

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because γ -thrombin (a degraded form of thrombin in which exosite 1 is cleaved) and Quick 1 dystrombin (a naturally occurring thrombin mutant with Arg 67 within exosite 1 replaced by Cys) fail to bind, whereas RA-thrombin (an exosite 2 mutant (Ye, J., et al. (1994) *J. Biol. Chem.* 269:17965-17970) with decreased affinity for heparin because Arg residues 93, 97, and 101 are replaced by Ala) binds to fibrin with an affinity similar to that of α -thrombin.

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1.3.2 Thrombin/Fibrin Interactions in the Presence of Heparin:

When heparin is present, the amount of thrombin that binds to fibrin changes, as does the mode of thrombin interaction with fibrin. With heparin concentrations up to 250 nM, the amount of thrombin that binds to fibrin increases (Figure 1A) as does the apparent affinity of thrombin for fibrin (Figure 1B); at 10 higher heparin concentrations, however, thrombin binding (Figure 1A) and the affinity of thrombin for fibrin progressively decrease (Figure 1B). These data extend the results of Hogg and Jackson who demonstrated enhanced thrombin binding to fibrin with fixed concentrations of heparin (see, Hogg, P.J., et al., *J. Biol. Chem.* 265:241-247 (1990)).

The mode of thrombin binding also changes in the presence of heparin. Whereas thrombin binds to fibrin via exosite 1 in the absence of heparin, enhanced α -thrombin binding seen in the presence of heparin is mediated by exosite 2 because heparin augments the binding of γ -thrombin to the same extent as α -thrombin but has little effect on the binding of RA-thrombin (Figure 2). Furthermore, excess α -thrombin bound in the presence of heparin is displaced with an antibody to exosite 2 or with prothrombin fragment 2 (F2) which, like heparin, also binds to exosite 2 (Arni, R.K., et al. (1993) *Biochemistry* 32:4727-4737). In contrast, hirugen, a synthetic analogue of the C-terminal of hirudin (Maraganore, J., et al. (1989) *J. Biol. Chem.* 264:8692-8698), has no effect on heparin-dependent binding of thrombin (Figure 3).

Such findings are interpreted as indicating ternary fibrin-thrombin-heparin complex formation wherein thrombin binds to fibrin directly via exosite 1, and heparin binds to both fibrin and exosite 2 on thrombin (Figure 4). This occurs because the affinity of heparin for fibrin ($K_d = 180$ nM) is similar to its affinity for α -thrombin ($K_d = 120$ nM). Heparin's interaction with fibrin is pentasaccharide-independent because heparin chains with low affinity for antithrombin bind as tightly as high affinity chains. The biphasic effect of heparin on thrombin binding (Figure 1) supports the concept of ternary complex formation. Thus, heparin promotes thrombin binding to fibrin until the heparin binding sites are saturated. With higher heparin concentrations, thrombin binding decreases as nonproductive binary fibrin-heparin and 30 thrombin-heparin complexes are formed.

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1.3.3 Consequences of Thrombin/Fibrin Interactions:

Thrombin within the ternary fibrin-thrombin-heparin complex is protected from inactivation by both antithrombin and heparin cofactor II (HCII). HCII is a naturally occurring antithrombin found in plasma that serves as a secondary inhibitor of thrombin. Thus, the heparin-catalyzed rate of thrombin inactivation by antithrombin or HCII is decreased in the presence of fibrin monomer (Figure 5). Over a wide range of heparin concentrations, the rates of inactivation by antithrombin and HCII in the presence of saturating amounts of fibrin monomer are up to 60- and 250-fold slower, respectively, than they are in its absence (Figures 6A and 6B). For protection to occur, both exosites must be occupied; exosite 1 by fibrin and exosite 2 by heparin. Thus, even though heparin enhances the binding of γ -thrombin and Quick 1

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dysthrombin to fibrin by binding to their intact exosite 2 and bridging them to fibrin, neither is protected from inactivation because their altered exosite 1 fails to interact with fibrin (Figure 7). RA-thrombin is susceptible to inactivation because even though it binds to fibrin with an affinity similar to that of α -thrombin, it has reduced affinity for heparin because of mutations at exosite 2 (Figure 7).

5 **1.3.4 Evidence that Thrombin Within the Ternary Fibrin-Thrombin-Heparin Complex Undergoes Allosteric Changes at the Active Site:**

Allosteric changes in the active site of thrombin induced by ternary complex formation likely reduce thrombin reactivity with its substrates or inhibitors. In support of this concept, it has been shown that the rate of thrombin-mediated cleavage of a synthetic substrate is increased when IIa is bound within the 10 ternary fibrin-thrombin-heparin complex, but not with binary thrombin-heparin or thrombin-fibrin complexes (Figure 8).

Example 2

2.0 Development of Medium Molecular Weight Heparin:

To catalyze thrombin inhibition, heparin bridges antithrombin to thrombin (Danielsson, A., *et al.* (1986) *J. Biol. Chem.* 261:15467-15473). Provision of this bridging function requires heparin chains with a minimal molecular weight of 5,400 (Jordan, R.E., *et al.* (1980) *J. Biol. Chem.* 225:10081-10090). Because the majority of LMWH molecules are < 5,400 Da, LMWH has little inhibitory activity against thrombin (Jordan, R.E., *et al.* (1980) *J. Biol. Chem.* 225:10081-10090). Since heparin bridges thrombin to fibrin to form the ternary fibrin-thrombin-heparin complex, it was hypothesized that this function also requires heparin chains of minimum molecular mass. Further, it was postulated that if this minimum molecular mass is different from that needed to bridge antithrombin to thrombin, there may be a window wherein the heparin chains are too short to bridge thrombin to fibrin, but are of sufficient length to bridge antithrombin to thrombin, thereby overcoming an important mechanism of heparin resistance.

It has now been discovered that such a window exists. For instance, the MMWH compositions of the present invention are long enough to catalyze thrombin inhibition by antithrombin, but do not promote thrombin binding to fibrin (Figure 9). In contrast to heparin, therefore, the rate of MMWH-catalyzed thrombin inhibition by antithrombin or HCII is almost the same in the presence of fibrin as it is in its absence (Figure 10).

2.1 Characteristics of Medium Molecular Weight Heparin:

Because the chains of MMWH are of sufficient length to bridge antithrombin to thrombin, the anti-factor IIa (*i.e.*, the ability of MMWH to catalyze or activate factor IIa (thrombin) inhibition by antithrombin) is the same as its anti-factor Xa activity (*i.e.*, the ability to catalyze factor Xa inhibition by antithrombin). In contrast, LMWH has greater anti-factor Xa activity than anti-factor IIa activity because more than half of the chains of LMWH are too short to bridge antithrombin to thrombin. Although unfractionated heparin also has equivalent anti-factor Xa and anti-factor IIa activity, it differs from the MMWH compositions of the invention in that it cannot catalyze thrombin inactivation in the presence of fibrin because the chains of unfractionated heparin are long enough to not only bridge antithrombin to thrombin, but also to bridge thrombin to fibrin.

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In its typical configuration, the specific activity of the MMWH compositions of the invention is similar to that of unfractionated heparin. Thus, its anti-factor Xa and anti-factor IIa activity may range from 90 to 150 U/mg and 40 to 100 U/mg, respectively. LMWH typically has a specific anti-factor Xa activity of 100 U/mg, whereas its anti-factor IIa activity ranges from 20 to 50 U/mg, depending on the molecular weight profile of the particular LMWH preparation.

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Example 3

Comparison of the Efficacy and Safety of the MMWH compositions of the Present Invention with Other Known Anticoagulants

This example illustrates a study comparing the efficacy and safety of a MMWH composition of the present invention, which is denoted in the figures as V21, LMWH, heparin and hirudin in the rabbit arterial thrombosis prevention model. The results indicate that the MMWH compositions of the present invention are more effective than LMWH and heparin and safer than hirudin. The arterial thrombosis prevention model was modified so that both efficacy and safety could be assessed in the same animal. Efficacy was assessed by measuring flow over 90 minutes distal to a 95% stenosis in an injured rabbit aorta, and safety was assessed by measuring blood loss over 30 minutes using the rabbit ear model. The four compounds were compared at three dosage levels. Each compound was administered as a bolus and infusion for 90 minutes. The doses listed in the following figures represent the bolus and infusion/60 minutes, administered for 90 minutes. The doses for heparin are shown as units/Kg, for LMWH and V21 as mg/Kg and for hirudin as mg/Kg. V21 has similar anti-Xa activity to LMWH and about twice the anti-IIa activity of LMWH. Thus, the specific activity of LMWH is 100 anti-Xa units/mg and 30 anti-IIa units/mg. The specific activity of V21 is 100 anti-Xa units/mg and 60 anti-IIa units/mg, whereas the specific activity of heparin is about 150 anti-Xa units and 150 anti-IIa units/mg. The anticoagulants were compared in the following dosages. Heparin 50 units/Kg and 75 units/Kg; LMWH and V21 0.5, 1.0 and 1.5 mg/Kg; Hirudin 0.1/0.1, 0.1/0.2 and 0.1/0.3 mg/Kg.

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For comparative purposes, 50 units of heparin is equivalent to 0.5 mg of LMWH or V21 in terms of anti-Xa activity, but has more than twice the anti-IIa activity of 0.5 mg of V21 and about 4 times the anti-IIa activity of LMWH. For equivalent anti-Xa activity, V21 has about twice the anti-IIa activity of LMWH.

The results obtained during this study are set forth in Figures 11, 12 and 13. Figure 11 compares the efficacy of the four anticoagulants using cumulative time that the aorta remained patent over the 90 minutes of observation as the outcome measure of efficacy. One hundred percent accumulated patency reflects complete patency and 0% cumulative patency reflects immediate and sustained thrombotic occlusion. The stenosed aorta clotted immediately and remained occluded for the full 90 minutes in the control animals, in the rabbits treated with low dose heparin (50/50 unit/Kg) and low dose LMWH (0.5/0.5 mg/Kg). There was a dose response with all four anticoagulants. However, the model was resistant to the antithrombotic effects of heparin and LMWH. Thus, both heparin in a dose of 75/75 units/Kg and LMWH in a dose of 1.0 mg/1.0 mg/Kg were ineffective (percent cumulative patency of 14% and 2% respectively), and LMWH 1.5/1.5 mg/Kg showed only limited effectiveness (38% cumulative patency). In contrast, the model was very responsive to the antithrombotic effects of V21 and hirudin. Thus, V21 at a dose of 0.5/0.5



mg/Kg was more effective than heparin at a dose of 75/75 units/Kg and more effective than LMWH in doses of 1.0/1.0 mg/Kg and 1.5/1.5 mg/Kg. Thus, V21 was at least three fold more potent than LMWH.

Figure 12 illustrates the effects of the four anticoagulants on 30 minute blood loss. A dose response was observed with LMWH, V21, and hirudin. At doses that showed greater efficacy, V21 was much safer than LMWH, and at doses that showed equivalent efficacy, V21 was safer than hirudin. V21 was also much more effective than heparin at doses that produced a similar degree of blood loss.

The comparative safety and efficacy of V21 and LMWH is illustrated in Figure 13. Based on the data (*i.e.*, three animals in each group), V21 appears to about 4 times more potent than LMWH on a weight basis. Therefore, for equivalent anti-Xa activity, V21 is 4 times more potent than LMWH, and for equivalent anti-IIa activity, V21 is about twice as potent. Such data support the importance of fibrin-bound thrombin in promoting thrombogenesis, since V21 is more effective against fibrin-bound thrombin than LMWH or heparin. At doses of 0.5 mg/Kg and 1.0 mg/Kg, V21 appears to be as safe as LMWH (although it is much more effective), but at a dose of 1.5 mg/Kg, LMWH produced much more bleeding than V21. Thus, V21 appears to have a more favorable efficacy to safety profile than LMWH.

15 **Example 4**

Studies Comparing the MMWH compositions of the Present Invention (V21) with LMWH

The efficacy of V21 (lot # D32) has been compared with LMWH (Enoxaparin) in both a heparin-sensitive and heparin-resistant thrombosis model in rabbits. The heparin-sensitive model is a venous thrombosis prophylaxis model and the heparin-resistant model is a venous thrombosis treatment model. V21 and LMWH have similar effects ex-vivo on the anti-factor Xa level and on the APTT (Figures 14 and 15).

Therefore, the two anticoagulants were compared on a gravimetric basis.

1.1 Prophylaxis Model

a. *Method:* Twenty seven male New Zealand White rabbits weighing between three and four kilograms are randomized into 3 treatment groups.

b. *Anaesthesia:* Anaesthesia is induced by a mixture of intramuscular ketamin (50 mg/kg) and xylazine (2 mg/kg) and maintained by isoflurane (1-3 %) and oxygen (1L/min).

c. *Surgical Procedure:* The ventral cervical area is shaved and two 22-gauge catheters (Becton-Dickinson, Sandy, UT) are inserted into the left central auricular artery and the marginal auricular vein for blood sampling and for intravenous administration of treatments. The right facial and external jugular vein are exposed through the ventral cervical skin incision. A 2 cm segment of the jugular vein is isolated from surrounding tissues and side branches are ligated using 4-0 silk suture. At this time control arterial blood sample is collected (1.8 ml of blood into 0.2 ml of 3.6% sodium citrate). Blood samples are spun and plasma stored in

35 -70°C for blood coagulation studies (A.PTT, TCT, and anti-Xa). Intravenous bolus of I-125 labelled rabbit fibrinogen (10 µL. ~ 1.000.000.00 CPM) is administered. Thereafter rabbits are randomized to one of the following iv treatments:

1. Saline (n=9) iv bolus of 1ml of saline

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2. Low molecular weight heparin (n=9)(Lovenox, enoxaparin sodium, lot # 923, Rhone-Poulenc Rorer, Montreal, Quebec, Canada) in a dose of 0.50 mg/kg (n3), 1.00 mg/kg (n3), 1.5 mg/kg (n=3).
3. V-21 (n=9) (D-32, lot # 521982132) in a dose of 0.50 mg/kg (n=3), 1.00 mg/kg (n=3), and 1.5, mg/kg (n=3).

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Four minutes after the treatment administration the right jugular vein is damaged in the length of 2 cm by 15 passages of the inflated balloon catheter (#4, Fogarty thrombectomy catheter). The balloon catheter is introduced into the right jugular vein via the right facial vein. Right after balloon vein injury, the catheter is withdrawn and a second arterial blood sample is taken. In addition, 1 ml of blood is also collected to measure radioactivity. Blood stasis is then induced within the 2 cm right jugular vein segment by placing two tourniquets around the vein. After 15 minute occlusion the jugular vein segment is excised and opened onto a pre-weighed square and weighed. Thereafter, the third arterial blood sample is collected for blood coagulation assay analysis.

d. End-points:

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Clot weight (%) is calculated as a percentage of blood by weight trapped in the venous segment. Clot radioactivity (%) is calculated as a percentage of 1 ml of whole blood radioactivity. Plasma samples were analyzed for APTT, TCT and anti-Xa.

Schematic diagram of the procedure is shown in appended Figure 16.

e. Results:

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As shown in Figures 16, 17 and 18 (for clot weight) and Figures 19, 20 and 21 (clot radioactivity), both agents are effective in this heparin-sensitive model, but V21 produces a steeper dose response and is more effective than LMWH at the two higher doses.

1.2 Treatment of Deep Vein Thrombosis in Rabbit Model

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a. 24 Hour Follow-up

The purpose of this study was to compare the efficacy of V-21 with LMWH both administered subcutaneously in the rabbit model of deep vein thrombosis.

Twenty four specific pathogen free, New Zealand White, male rabbits (3-4 kg of b. wt.) were anesthetized by intramuscular injection of ketamin (50 mg/kg) and xylazine (2 mg/kg). The ventral cervical area was shaved and prepped with alcohol and iodine solution. Venous and arterial catheters were inserted into the left central auricular artery and the marginal auricular vein using an 22 gauge intravenous catheter (Angiocath, Becton Dickinson Vascular Access, Sandy, Utah., USA) for blood sample collection, and for intravenous administration of fluids and anticoagulants. Rabbits were transferred into an operating room and maintained on inhalation anesthesia which consisted of a mixture of isoflurane (1-4%), oxygen (1 L/min) and nitrous oxide (0.5 L/min) delivered by a face mask.

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b. Clot Formation: The right external jugular and the facial vein were exposed through the ventral cervical skin incision. Segmental occlusion of the facial vein was achieved by two No 4-0 silk suture placed 0.5 centimetres apart. All side branches of the jugular vein were ligated in the length of 4 centimetres. Fogerty thrombectomy catheter (#4 Fr) was introduced into the jugular vein via the facial vein and inflated. Four centimetres of the jugular vein was damaged by 15 passages of inflated balloon catheter and then the

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catheter was withdrawn. A 1.5 centimetres occluded jugular vein segment was created using two 4-0 silk sutures placed around the damaged vein and then emptied using finger compression. One millilitre of arterial blood was drawn from the central auricular artery into the 1 ml syringe and mixed in a sterile tube with approximately 1 μ Ci of iodine-125 labelled rabbit fibrinogen. 0.6 millilitres of the radiolabelled blood was then drawn from the tube into a 1 ml syringe and the first 0.4 ml of labelled blood was equally divided into two tubes and left to clot. The remaining 0.2 ml was then injected into the occluded jugular vein segment via the home made cannula (23 gauge needle connected to PB #60). Clots generated in the test tubes served as baseline values for clot weight and radioactivity. Pilot studies have shown that there was around 5 % difference in clot weight or radioactivity between the clots generated in tubes and in the jugular vein. The thrombus generated in the jugular vein was left to mature for 30 minutes and the facial vein was ligated. Twenty five minutes into the thrombus maturation rabbits were randomized to receive:

- 1) saline treatment (n=4) 1 ml of sterile saline BID sc;
 - 2) low molecular weight heparin (Enoxaparin sodium, Lovenox lot #923, Rhone-Poulenc Rorer, Montreal, Quebec) at a dose of 1 mg/kg BID, sc (n=4), and 3 mg/kg BID sc (n=4); and
 - 3) V-21 (D-32, lot # 521982132) at a dose of 1 mg/kg BID sc (n=4) and at 3 mg/kg BID sc (n=4).

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Thirty percent of the first dose was administered intravenously and 70 % subcutaneously; the second dose was given only subcutaneously. Just prior to tourniquet removal at 30 minutes, the thrombus was fixed to the vein wall by two silk sutures to prevent its migration in the post-operative period. There was no residual stenosis of the jugular vein left after tourniquet removal. The cervical incision was closed in a routine manner. Rabbits were left to recover breathing 100% oxygen and then transferred to the recovery room. All rabbits were euthanized at the 24 hour time interval.

c. **Blood collection:** Arterial blood was collected prior to surgery (control) and at 5 minutes, 1, 3, 6, 9, 12, and 24 hours after clot maturation. At each time interval 2 millilitres of citrated blood was collected (9:1 ratio, 3.8% sodium citrate) for APTT, TCT and Xa assays. Blood loss was replaced by iv administration of saline.

d. End-points: Using thrombus weight in milligrams (AG Balances #104, Mettler Toledo, Fisher Scientific Limited, Whitby, Ontario) and thrombus radioactivity (CPM) at the time of clot induction (clot created in tubes) and at 24 hours the following end-points were calculated:

Percentage change in clot weight (PCCW) was calculated using clot

weight generated in a test tube at the time of surgery divided by clot weight generated in a test tube times 100.

Clot accretion (CA) at % was calculated as follows: AC=PCCW-CL

e. **Results:** As illustrated in Figures 23-28, V21 is more effective than LMWH in this heparin-resistant model.

Example 5

Preparation of the MMWH compositions of the Present Invention by a Limited Periodate Oxidation/Hydrolysis of Heparin

1.1 Study of Limited Periodate Oxidation/Hydrolysis of Heparin

Heparin was dissolved in deuterated water to make 10% of stock solution. Sodium periodate was dissolved in deuterated water to make 100 mM stock solution and kept at 4°C. The periodate oxidation reaction was carried out at 2.5% of heparin concentration with increasing sodium periodate concentration, 1 mM, 2.5 mM, 5 mM, 8 mM, 10 mM, and 20 mM, at room temperature for about 18 hours. The reaction was stopped by adding 50 mM of ethylene glycol and incubation for 30 minutes. Then, the reaction mixture was brought to 0.25 N NaOH and incubated at room temperature for 3 hours. After the reaction, the pH was adjusted to pH 7 by 6 N HCl. An aliquot of each reaction mixture was run on an HPLC-GPC (G2000 column, 0.5 ml/min, injection volume 20 µl) for molecular weight analysis. The molecular weight profiles of the reaction at sodium periodate concentrations of 5 mM, 8 mM, 10 mM, and 20 mM decrease in comparison to heparin with increasing sodium periodate concentration. The result indicated that the desired cleavage can be achieved using sodium periodate concentrations of between about 5 mM and about 20 mM, and at room temperature for about 18 hours. The study (not shown) indicated that the best alkaline hydrolysis can be achieved using 0.25 N NaOH, at room temperature for 3 hours. Thus, the reaction conditions used in this experiment are called "limited periodate/hydrolysis" conditions.

1.2 Preparation of MMWH compositions of the Present Invention by Limited Periodate Oxidation/Hydrolysis

100 mg of heparin was treated using the limited periodate/hydrolysis conditions, 7 mM sodium periodate, and purified by P30 gel-filtration chromatography. 30 mg of final product, *i.e.*, V2I-D32, was obtained having a molecular weight ranging from about 6,000 Daltons to about 12,000 Daltons, and having a peak molecular weight of about 9,000 Daltons.

Example 6

Studies were undertaken to select MMWH compositions with an optimal molecular weight range and to identify a manufacturing process which could be readily scaled up to obtain a heparin fraction within this range.

A molecular weight range between 6,000 and 10,000 was selected as an optimal molecular weight range. Compositions with a minimum molecular weight of 6,000, which corresponds to 20 saccharide units, should provide heparin chains that have pentasaccharide-containing chains long enough to bridge antithrombin to thrombin. With a maximum molecular weight of 10,000, which corresponds to 33 saccharide units, the chains will (a) be too short to bridge thrombin to fibrin, a phenomenon that requires chains of 40 saccharide units or more, and (b) too short to exhibit non-specific binding to plasma proteins, a phenomenon that occurs with chains of 30 saccharide units or more.

Heparin Fractions:

Unfractionated heparin was depolymerized with heparinase, nitrous acid, or periodate to yield fractions of approximately 6,000, 8,000, and 10,000 Da. While initial fractions produced by these three methods were polydispersed, more size-restricted fractions of these molecular weights were prepared using either heparinase or nitrous acid depolymerization. The characteristics of these fractions are illustrated in Table 1 along with their specific anti-Xa and anti-IIa activities.

Affinities for Antithrombin

The affinities of each of the heparin fractions for antithrombin was determined as previously described (Weitz et al, Circulation 1999;99:682-689). Briefly, a 1 X 1 cm. quartz cuvette containing 100 nM antithrombin in 2 ml of 20 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) was excited at 200 nm (6-nm slit width) and intrinsic fluorescence was continuously monitored in time drive at 340 nm (6-nm slit width) with a Perkin-Elmer LS50B luminescence spectrometer. The contents of the cuvette were stirred with a micro-stir bar and maintained at 25°C with a recirculating water bath. Intrinsic fluorescence intensity was measured before (I_0) and after (I) addition of 5 to 10 ml of 10-mg/ml solutions of the various heparin fractions. Titrations were continued until there was no change in I . After the experiment, I values were read from the time drive profile and I/I_0 values were calculated and plotted versus heparin concentration. The data were then analyzed as described previously (Weitz et al, supra). From this analysis, stoichiometry can be obtained which is interpreted as indicating the proportion of pentasaccharide-containing chains within each heparin fraction.

The affinities are summarized in Table 2. Also illustrated is the estimated percentage of pentasaccharide-containing chains within each fraction. Fractions prepared by either heparinase or nitrous acid depolymerization exhibit similar affinities for antithrombin. Although the 10,100 Da fraction prepared by periodate depolymerization exhibits affinity for antithrombin similar to that of heparinase or nitrous acid-derived fractions, the lower molecular weight periodate-derived fractions have lower affinities consistent with their reduced anti-IIa and anti-Xa activities (Table 1). As might be expected, regardless of the method used for depolymerization, the percentage of pentasaccharide-containing chains increases as the mean molecular weight increases.

As controls for these analyses, unfractionated heparin, high and low affinity fractions of heparin prepared by affinity chromatography using an antithrombin column, enoxaparin, and synthetic pentasaccharide also were studied. As illustrated in Table 3, the high affinity fraction of heparin and synthetic pentasaccharide exhibit the highest affinity for antithrombin. Only these two preparations have 100% pentasaccharide-containing chains.

Affinities for Thrombin:

The affinities of the polydispersed heparinase, nitrous acid, and periodate-derived heparin fractions for thrombin were measured as described above except thrombin was used in place of antithrombin (Fredenburgh, JC et al. J. Biol. Chem. 1997;272:25493-25499). As illustrated in Table 4, when affinities are expressed in $\mu\text{g}/\text{ml}$, all fractions exhibited similar affinities for thrombin.

Heparin-catalyzed rates of thrombin inhibition by antithrombin in the absence or presence of fibrin monomer:

The second order rate constants for thrombin inhibition by antithrombin were measured in the absence or presence of the various heparin fractions in concentrations ranging from 0 to 600 $\mu\text{g}/\text{ml}$. Heparin-catalyzed rates of thrombin inhibition by antithrombin were measured both in the absence or presence of 4 μM fibrin monomer. The fibrin monomer was prepared as previously described, and the data were analyzed as described elsewhere (Becker DL et al, J. Biol. Chem. 1999;274:6226-6233).

The inhibitory effect of fibrin-monomer on the rates of inhibition of thrombin by antithrombin is

shown with the heparinase (Figure 29), nitrous acid (Figure 30), and periodate-derived heparin fractions (Figure 31). The background inhibition with fibrin monomer is 6-fold as determined by measuring the inhibitory effect of fibrin monomer on the heparin-catalysed rate of factor Xa inactivation by antithrombin. (Figures 32 and 33). There is less reduction in the rate of thrombin inactivation by antithrombin with the heparinase or nitrous acid-derived heparin fractions than with unfractionated heparin. In contrast, greater inhibition with fibrin monomer is seen with the periodate-derived heparin fractions (Figure 31). With the size-restricted heparinase -derived fractions, fibrin-monomer produces no more than background inhibition.

Heparin-catalyzed inhibition of factor Xa by antithrombin:

The second order rate constants for factor Xa inhibition by antithrombin were measured in the absence or presence of the various heparin fractions in concentrations ranging from 0 to 1,500 µg/ml as described elsewhere (Becker et al, supra). The results for the heparinase, nitrous acid, and periodate-derived fractions are illustrated in Figures 34 to 36, respectively. When added in gravimetrically equivalent amounts, all of the heparin fractions produce less catalysis of factor Xa inhibition by antithrombin than unfractionated heparin.

Augmentation of thrombin binding to fibrin:

¹²⁵I-labeled thrombin binding to fibrin was measured in the absence or presence of the various heparin fractions in concentrations ranging from 0 to 7,500 nM as previously described (Becker et al, supra). Unfractionated heparin was used as a control in these experiments. The results with heparinase, nitrous acid, and periodate-derived heparin fractions are illustrated in Figures 37 to 39, respectively. Regardless of the method of depolymerization, the 10,000 Da fractions augment thrombin binding to fibrin to a greater extent than the lower molecular weight fractions. This is best illustrated with the more size-restricted heparinase or nitrous acid-derived fractions (Figures 40 and 41, respectively).

Antithrombotic activity of heparin fractions:

An extracorporeal circuit was used to compare the antithrombotic activity of the heparin fractions. As previously described (Weitz et al, supra), different concentrations of each of the heparin fractions was added to recalcified human whole blood spiked with ¹²⁵I-labeled human fibrinogen and maintained at 37°C in a water bath. A peristaltic pump was then used to circulate the blood through a 40 µ blood filter. Clotting of blood within the filter was detected by (a) measuring pressure proximal to the filter with an in-line pressure gauge, and (b) removing serial blood samples from the reservoir and counting residual radioactivity as an index of fibrinogen consumption. Starting activated clotting times also were measured.

As illustrated in Table 5, regardless of the method of depolymerization, fractions of 10,000 Da were effective at a concentration of 10 µg/ml. Thus, filter patency was maintained during the 90 min observation period and fibrinogen consumption was less than 10%. At a concentration of 10 µg/ml, the heparinase-derived 8,000 Da fraction was effective. The 6,000 Da heparinase fraction was effective at 14 µg/ml. Although patency was maintained with 14 or 16 µg/ml of the 5,600 Da nitrous acid-derived fractions, fibrinogen consumption was 33 and 20%, respectively. As a control, enoxaparin was also evaluated. This drug was ineffective at 10 or 20 µg/ml with filter failure occurring at 30 and 55 min, respectively.

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The antithrombotic activities of the more size-restricted heparinase and nitrous acid-derived heparin fractions are illustrated in Table 6. All fractions were tested at a concentration of 10 µg/ml with 10 µg/ml enoxaparin serving as a control. Except for the 5,300 Da heparinase-derived fraction, all of the heparin fractions were effective in maintaining patency for > 90 min and reducing fibrinogen consumption to < 10%.

5 In contrast, enoxaparin was ineffective with filter failure occurring at 30 min and fibrinogen consumption of 73%. The antithrombotic activities of the more size-restricted heparinase, nitrous acid-derived heparin and periodate fractions are illustrated in Table 7. All fractions were tested at a concentration of 10 µg/ml with 10 µg/ml enoxaparin serving as a control. The heparinase and nitrous acid derivative fractions were effective in ameliorating patency. The periodate-derived fraction and enoxaparin were less effective.

10 It is to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reading the above description. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled. The disclosures of all articles and references, including patent applications and publications, are incorporated herein by reference in their entirety for all purpose.

15

TABLE 1
CHARACTERISTICS OF THE HEPARIN FRACTIONS PROVIDED BY LEO

Depolymerization Method	Molecular Weight	Polydispersity	Anti-IIa	Anti-Xa
Heparinase	6,000	1.5	72	106
	8,500	1.5	100	134
	10,350	1.5	152	111
HN O ₂	5,600	1.5	59	118
	8,200	1.4	100	152
	10,300	1.4	119	180
IO ₄ ⁻	6,700	1.5	11	30
	7,900	1.5	19	43
	10,100	1.5	43	88
	10,300	1.5	42	84
Heparinase	5,300	1.2	22	81
	8,450	1.2	67	116
	9,750	1.3	87	155
HN O ₂	5,900	1.2	32	95
	7,700	1.3	84	123
	9,300	1.2	106	162

TABLE 2

AFFINITIES OF HEPARIN FRACTIONS FOR ANTITHROMBIN AND
PERCENTAGE OF PENTASACCHARIDE-CONTAINING CHAINS
IN EACH FRACTION

Glycosaminoglycan		K _d	Pentasaccharide-containing
		nM	%
Heparinase	6,000	91.2 ± 15.9	14.8
	8,050	61.7 ± 4.2	20.8
	10,350	48.0 ± 7.8	27.0
HN0 ₂	5,600	55.6 ± 0.2	16.0
	8,200	42.5 ± 9.1	25.2
	10,300	37.5 ± 2.9	31.5
IO ₄ ⁻	6,700	170.1 ± 27.4	6.8
	8,200	140.3 ± 4.5	10.5
	10,300	57.0 ± 28.3	25.6
Heparinase	5,300	421.6 ± 72.3	15.9
	8,450	167.0 ± 17.0	29.4
	9,750	138.4 ± 2.2	32.2
HN0 ₂	5,900	32.8 ± 0.3	21.1
	7,700	23.1 ± 4.1	26.9
	9,300	17.0 ± 0.3	36.6

TABLE 3

AFFINITIES OF UNFRACTIONATED HEPARIN, HEPARIN WITH HIGH
OR LOW AFFINITY FOR ANTITHROMBIN, ENOXAPARIN AND
SYNTHETIC PENTASACCHARIDE FOR ANTITHROMBIN AND
PERCENTAGE OF PENTASACCHARIDE-CONTAINING CHAINS
IN EACH PREPARATION

Glycosaminoglycan	K_d	Pentasaccharide-	%
		containing	
Unfractionated heparin	31.7		43.1
High affinity heparin	10.7		114
Low affinity heparin	6670.0		1.0
Enoxaparin	46.8		14.4
Pentasaccharide	31.0		102

TABLE 4

**AFFINITIES OF HEPARINASE AND NITROUS ACID-DERIVED
HEPARIN FRACTIONS FOR THROMBIN**

Glycosaminoglycans		K _d	K _d
		nM	μg/ml
Heparinase	6,000	1517 ± 196	9.1 ± 1.2
	8,050	872 ± 9	7.0 ± 0.1
	10,350	699 ± 97	7.2 ± 1.0
HN0 ₂	5,600	1288 ± 92	7.2 ± 0.5
	8,200	695 ± 37	5.7 ± 0.3
	10,300	632 ± 51	6.5 ± 0.5
I0 ₄	6,700	731 ± 159	4.9 ± 1.1
	7,900	587 ± 8	4.6 ± 0.1
	10,100	285 ± 5	2.9 ± 0.5

TABLE 5

**ANTITHROMBOTIC ACTIVITY OF HEPARINASE, NITROUS ACID, AND
PERIODATE-DERIVED HEPARIN FRACTIONS AND ENOXAPARIN
IN AN EXTRACORPOREAL CIRCUIT**

Glycosaminoglycan	Concentration	Time to Filter Failure	Fibrinogen Consumption	Starting
				μg/ml sec
Heparinase	6 kDa	8	75	82 271
		10	>90	68 248
		12	>90	31 226
		14	>90	7 335
	8 kDa	5	40	70 241
		6	45	70 230
		8	>90	54 256
		10	>90	6 282
	10 kDa	5	45	72 231
		6	>90	36 299
		8	>90	29 300
		10	>90	4 327
HN0 ₂	5.6 kDa	10	>90	29 238
		12	>90	57 235
		14	>90	33 238
		16	>90	20 264
	8.2 kDa	10	60	81 239
		11	>90	28 313
		12	>90	10 301
		16	>90	8 428
	10.3 kDa	8	>90	14 303
		10	>90	28 287
		11	>90	7 341
		12	>90	7 359
IO ₄ ⁻	6.7 kDa	10	45	68 —
	7.9 kDa	10	90	73 299
	10.1 kDa	10	>90	6 318
Enoxaparin	10	30	73	202
	20	55	64	231

TABLE 6

**COMPARISON OF ACTIVITY OF 10 µg/ml HEPARINASE, NITROUS
ACID- DERIVED HEPARIN FRACTIONS WITH ENOXAPARIN IN
EXTRACORPOREAL CIRCUIT**

Glycosaminoglycan	Time to Filter Failure	Fibrinogen Consumption	Starting
			ACT
	min	%	sec
Heparinase	5,300	30	205
	8,450	>90	283
	9,750	>90	312
HNNO ₂	5,900	>90	278
	7,700	>90	314
	9,300	>90	557
Enoxaparin		30	202

TABLE 7

COMPARISON OF ACTIVITY OF 10 µg/ml HEPARINASE, NITROUS ACID-DERIVED AND PERIODATE-DERIVED HEPARIN FRACTIONS WITH ENOXAPARIN IN EXTRACORPOREAL CIRCUIT

Glycosaminoglycan		Time to Filter Failure	Fibrinogen Consumption	Starting ACT
		min	%	sec
Heparinase	8,450	>90	7.1	289
NHNO ₂	7,700	>90	5.6	296
HO ₄	7,900	30	77	242
Enoxaparin		30	80	232

WE CLAIM:

1. A medium molecular weight heparin (MMWH) composition comprising a mixture of sulfated oligosaccharides having molecular weights ranging from about 6,000 Daltons to about 12,000 Daltons.
2. The MMWH composition in accordance with claim 1, wherein said MMWH composition (1) inhibits fibrin-bound thrombin and fluid-phase thrombin by catalyzing antithrombin, and (2) inhibits thrombin generation by catalyzing factor Xa inactivation by antithrombin.
- 5 3. The MMWH composition in accordance with claim 1, wherein said MMWH composition has an anti-factor IIa activity of about 40 U/mg to about 100 U/mg, and an anti-factor Xa activity of about 90 U/mg to about 150 U/m
- 10 4. The MMWH composition in accordance with claim 3, wherein said MMWH composition has an anti-factor IIa activity of about 60 U/mg to about 75 U/mg, and an anti-factor Xa activity of about 100 U/mg to about 125 U/mg.
- 5 5. The MMWH composition in accordance with claim 4, wherein said MMWH composition has an anti-factor IIa activity of about 65 U/mg, and an anti-factor Xa activity of about 115 U/mg
6. The MMWH composition in accordance with claim 1, wherein said MMWH composition comprises a mixture of sulfated oligosaccharides having molecular weights ranging from about 8,000 Daltons to about 10,000 Daltons.
7. The MMWH composition in accordance with claim 1, wherein said MMWH composition has an average molecular weight of about 9,000.
8. The MMWH composition in accordance with claim 1, wherein at least 31% of said sulfated oligosaccharides have a molecular weight greater than or equal to about 7,800.
9. The MMWH composition in accordance with claim 1, wherein at least 25% of said sulfated oligosaccharides have a molecular weight greater than or equal to about 10,000 Daltons.
10. A medium molecular weight heparin (MMWH) composition comprising a mixture of oligosaccharides derived from heparin characterized by one or more of the following characteristics:
 - (a) having antithrombin- and heparin cofactor II (HCII)-related anticoagulant activity *in vitro*;
 - (b) the oligosaccharides are too short to bridge thrombin to fibrin, but are of a sufficient length to bridge antithrombin or HCII to thrombin;
 - (c) having at least 15%, 20%, 25%, 30%, 35%, or 40% oligosaccharides with at least one or more pentasaccharide sequence;
 - 30 (d) enriched for oligosaccharides having a molecular weight range from about 6,000 to about 11,000; 7,000 to 10,000; 7,500 to 10,000; 7,800 to 10,000; 7,800 to 9,800; or 7,800 to 9,600; 8,000 to 9,600;
 - (e) the oligosaccharides have a mean molecular weight of about 7,800 to 10,000, preferably 7,800 to 9,800, more preferably 8,000 to 9,800;
 - 35 (f) at least 30%, 35%, 40%, 45%, or 50% of the oligosaccharides have a molecular weight greater than or equal to 6000 Daltons, preferably greater than or equal to 8000 Daltons;
 - (g) a polydispersity of 1.1 to 1.5, preferably 1.2 to 1.4, most preferably 1.3;

- (h) having similar anti-factor Xa and anti-factor IIa activities, preferably a ratio of anti-factor Xa activity to anti-factor IIa activity from about 2:1 to about 1:1 and, more preferably, from about 1.5:1 to about 1:1;
- (i) an anti-factor Xa activity from about 80 IU/mg to about 155 IU/mg, preferably 90 IU/mg to about 130 IU/mg, more preferably, from about 95 IU/mg to about 120 IU/mg and, most preferably 100-110 IU/mg; and
- (j) an anti-factor IIa activity from about 20 IU/mg to about 150 IU/mg; preferably 40 IU/mg to about 100 IU/mg, more preferably, from about 80 IU/mg to about 100 IU/mg, most preferably about 90-100 IU/mg.

- 5 11. A MMWH composition in accordance with claim 10 which has the characteristics of (a), (b), (c) and (d);
(a) (b), (c), and (e); (b), (c), (e), and (g); (b), (d), (c), (e), and (h); (b) (c), (d), and (g); (b), (e), (g), (i), and
(j); (b), (e), (f), (g), (i) and (j); or (a) through (j).
- 10 12. A MMWH composition in accordance with claim 10 enriched for oligosaccharides having a molecular weight range of 7,800 to 8,800, preferably 7,800 to 8,600, more preferably 7,800 to 8,500, most
15 preferably 8,000 to 8,500.
13. A MMWH composition in accordance with claim 10 enriched for oligosaccharides having a molecular weight range of 9,000 to 10,000, preferably 9,200 to 9,800, more preferably 9,300 to 9,600, most
preferably 9,400 to 9,600.
- 20 14. A MMWH composition in accordance with claim 10 comprising oligosaccharides having a mean molecular weight of 7,800 to 8,800, preferably 7,800 to 8,600, more preferably 7,800 to 8,500, most
preferably 8,000 to 8,500.
- 25 15. A MMWH composition in accordance with claim 10 comprising oligosaccharides having a mean molecular weight of 9,000 to 10,000, preferably 9,200 to 9,800, more preferably 9,300 to 9,600, most
preferably 9,400 to 9,600.
16. A MMWH composition as claimed in claim 10, 11, 12, 13, 14, or 15 derived from heparinase depolymerization or nitrous acid depolymerization of unfractionated heparin.
- 30 17. A method for treating a thrombotic condition in a subject comprising administering to the subject a pharmacologically acceptable dose of a medium molecular weight heparin (MMWH) composition as claimed in any of the preceding claims.
18. The method in accordance with claim 17, wherein said thrombotic condition is arterial thrombosis, coronary artery thrombosis, venous thrombosis, or pulmonary embolism.
- 35 19. The method in accordance with claim 17, wherein said MMWH composition is administered by injection.
20. A method of preventing the formation of a thrombus in a subject at risk of developing thrombosis comprising administering to the subject a pharmacologically acceptable dose of a medium molecular weight heparin (MMWH) composition as claimed in any of the preceding claims.
21. The method in accordance with claim 20, wherein the subject is at increased risk of developing thrombosis due to a medical condition which disrupts hemostasis.
22. The method in accordance with claim 21, wherein the medical condition is coronary artery disease, or atherosclerosis.

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23. The method in accordance with claim 20, wherein the subject is at increased risk of developing thrombosis due to a medical procedure.
24. The method in accordance with claim 23, wherein the medical procedure is cardiac surgery, cardiopulmonary bypass, catheterization, or atherectomy.
- 5 25. The method in accordance with claim 24, wherein the catheterization is cardiac catheterization
26. A method for inhibiting thrombus formation in a patient comprising the step of administering to the patient a pharmacologically acceptable dose of a medium molecular weight heparin (MMWH) composition as claimed in any of the preceding claims.
- 10 27. A pharmaceutical composition comprising a MMWH composition as claimed in any of the preceding claims and a pharmaceutically acceptable carrier.
28. A method for treating deep vein thrombosis in a patient comprising administering to a patient undergoing orthopedic surgery a therapeutically effective amount of a MMWH composition as claimed in any of the preceding claims.
- 15 29. A method for preventing a pulmonary embolism in a subject comprising administering to the subject a therapeutically effective amount of a MMWH composition as claimed in any of the preceding claims.
30. A method for preparing a medium molecular weight heparin (MMWH) composition comprising:
- (a) subjecting unfractionated heparin to a limited periodate oxidation reaction such that only the iduronic acids of the unfractionated heparin are oxidized;
 - (b) subjecting the oxidized unfractionated heparin of step (a) to alkaline hydrolysis; and
 - (c) recovering said MMWH composition, wherein the MMWH composition comprises a mixture of sulfated oligosaccharides having molecular weights ranging from about 8,000 Daltons to about 12,000 Daltons.
31. Use of a MMWH composition as claimed in any of the preceding claims in the preparation of a medicament for treating a thrombotic condition, or preventing the formation of a thrombus in a subject at risk of developing thrombosis.
- 20 32. Use of a MMWH composition as claimed in any of the preceding claims in the preparation of a medicament for inhibiting fibrin-bound thrombin and thrombin generation in a subject.
33. Use of a MMWH composition as claimed in any of the preceding claims in the preparation of a medicament for treating deep vein thrombosis.
- 25 34. Use of a MMWH composition as claimed in any of the preceding claims in the preparation of a medicament for preventing pulmonary embolism in a subject.

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(54) Title: HEPARIN COMPOSITIONS THAT INHIBIT CLOT ASSOCIATED COAGULATION FACTORS

(57) Abstract: The present invention provides compositions and methods for the treatment of cardiovascular diseases. More particularly, the present invention relates to modifying thrombus formation by administering an agent which, *inter alia*, is capable of (1) inactivating fluid-phase thrombin and thrombin which is bound either to fibrin in a clot or to some other surface by catalyzing antithrombin; and (2) inhibiting thrombin generation by catalyzing factor Xa inactivation by antithrombin III (ATIII). The compositions and methods of the present invention are particularly useful for preventing thrombosis in the circuit of cardiac bypass apparatus and in patients undergoing renal dialysis, and for treating patients suffering from or at risk of suffering from thrombus-related cardiovascular conditions, such as unstable angina, acute myocardial infarction (heart attack), cerebrovascular accidents (stroke), pulmonary embolism, deep vein thrombosis, arterial thrombosis, etc.

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(B) EFFECT OF HEPARIN ON THE AFFINITY OF α -THROMBIN FOR FIBRIN

HEPARIN (μ M)	Kd (μ M)
0	3.22
0.1	0.25
0.25	0.15
1	0.79
20	4.26

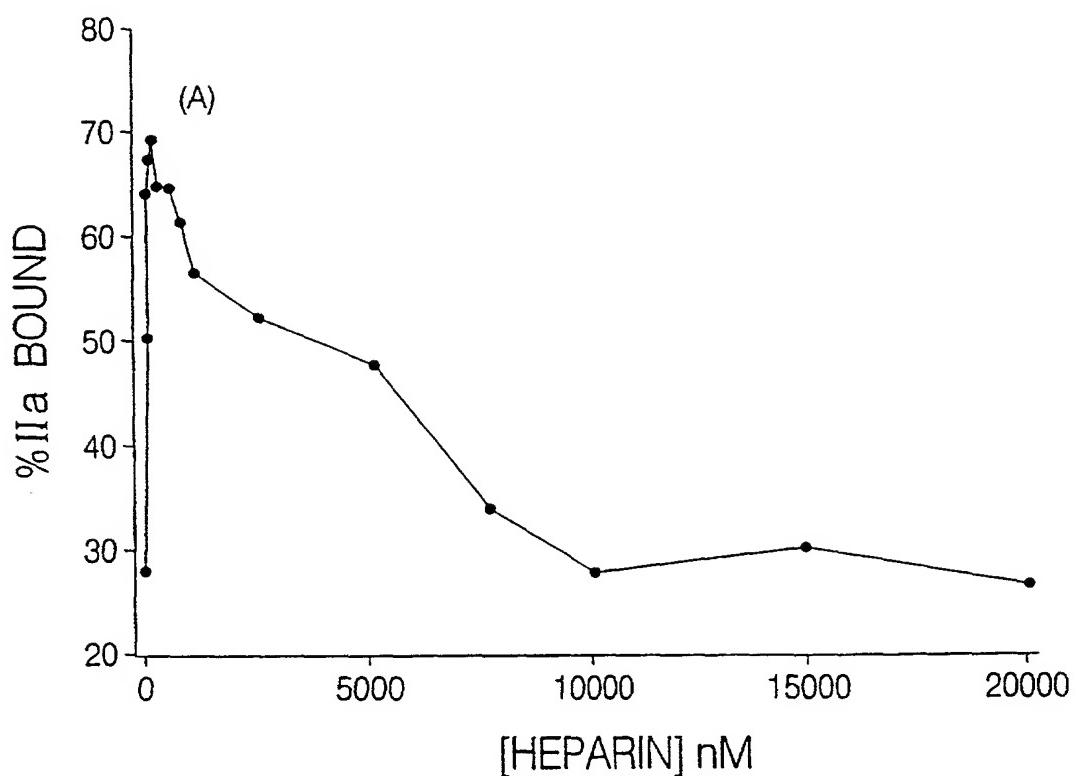


Fig. 1

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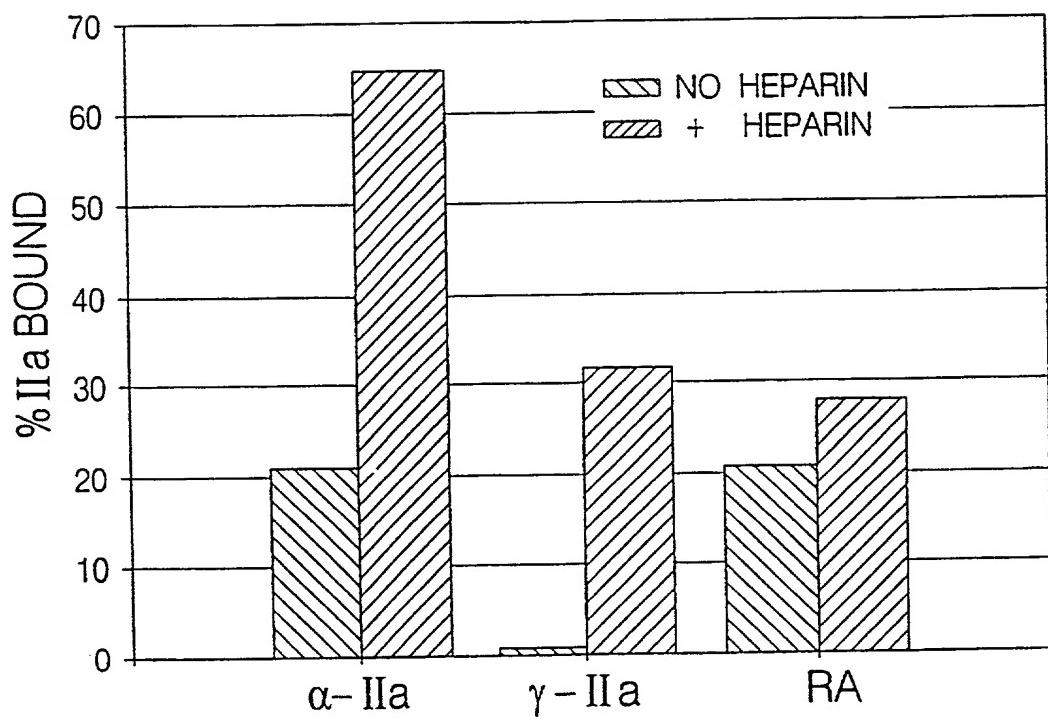


Fig. 2

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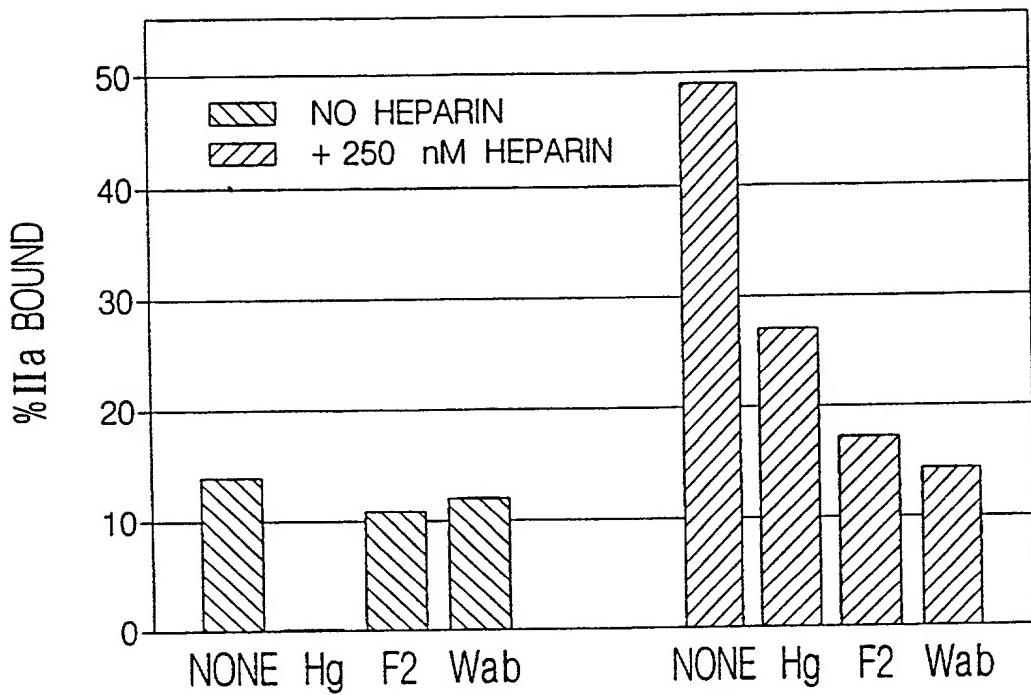


Fig. 3

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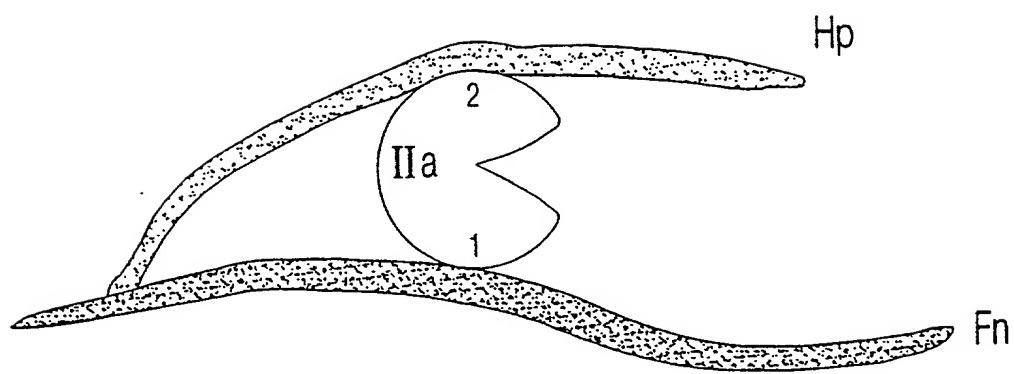


Fig. 4

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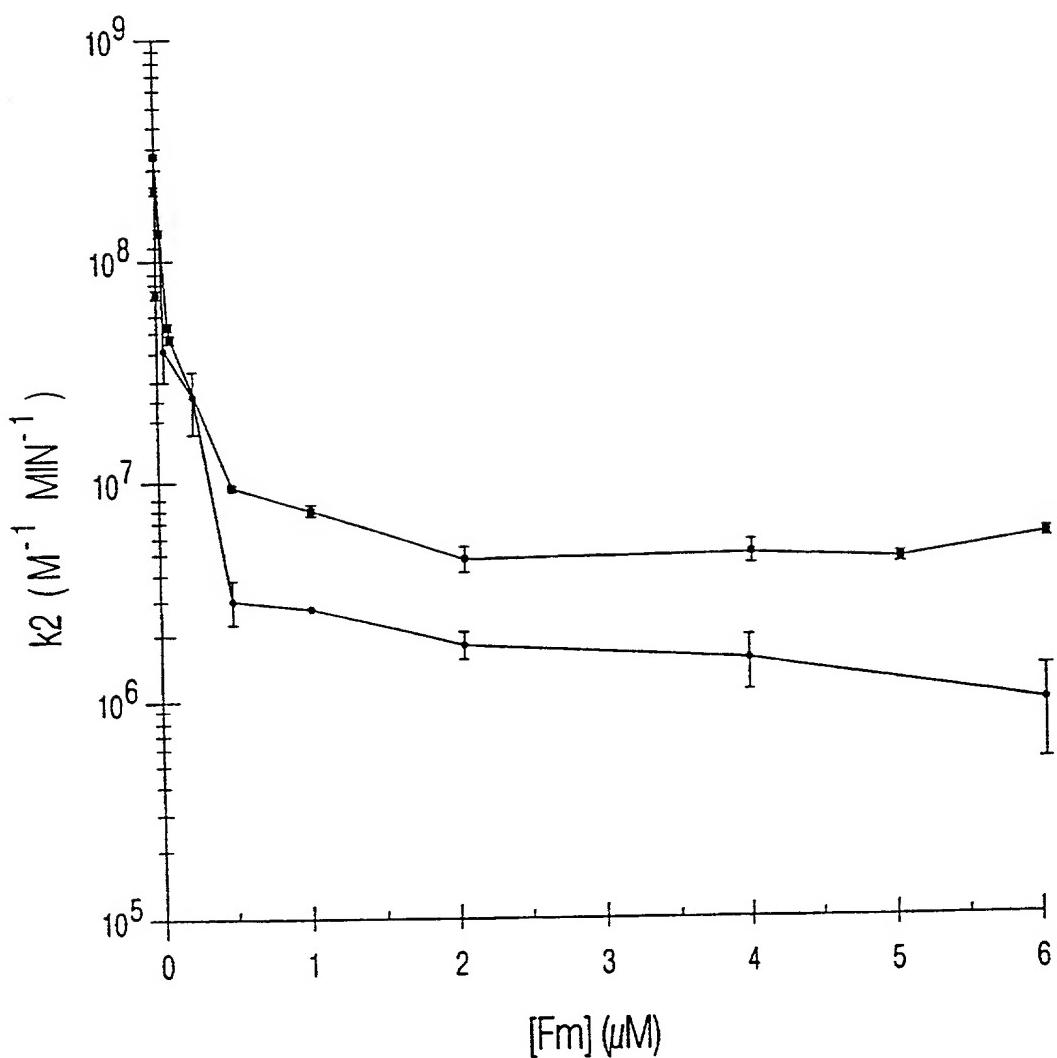


Fig. 5

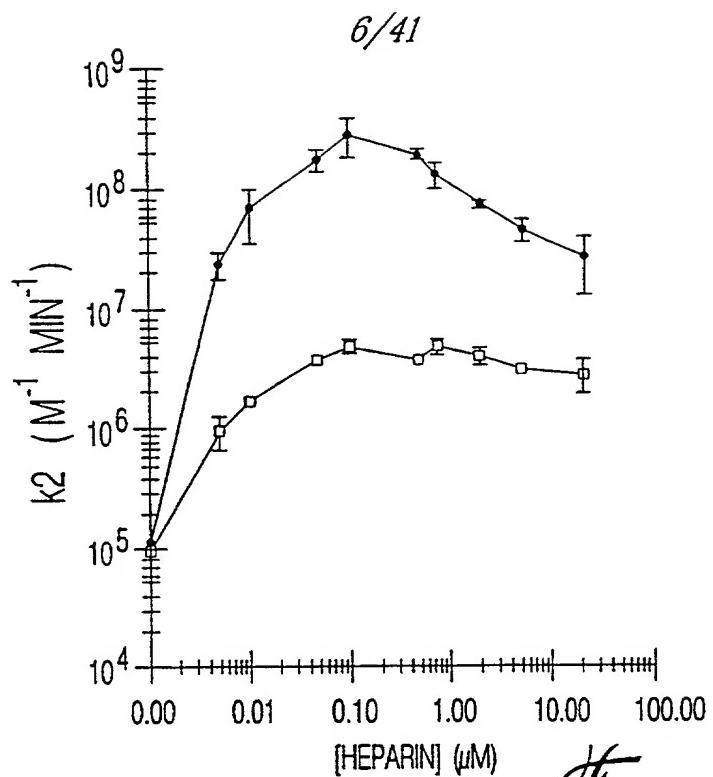


Fig. 6A

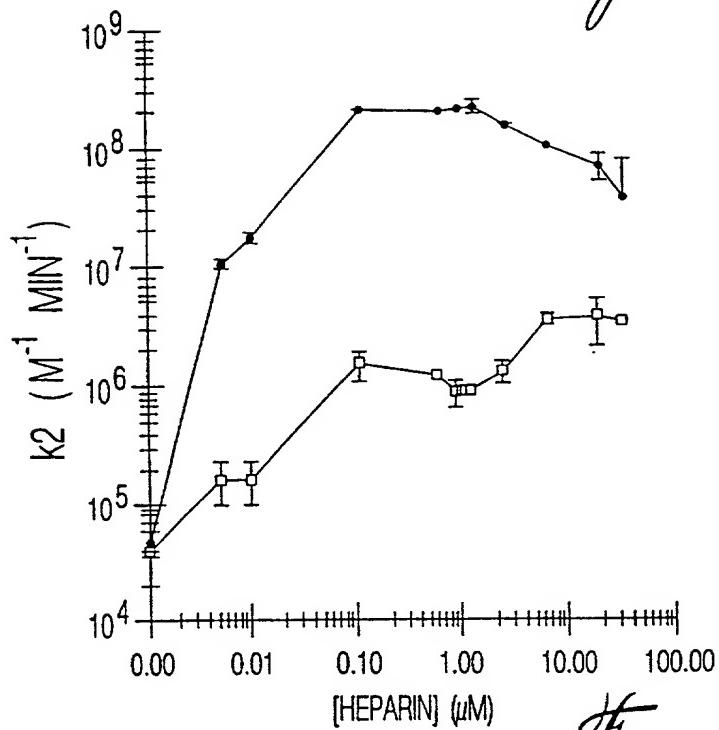


Fig. 6B

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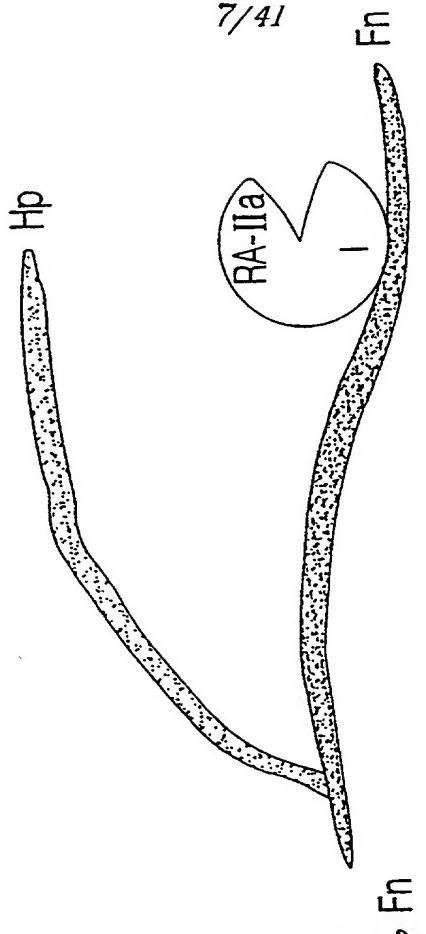
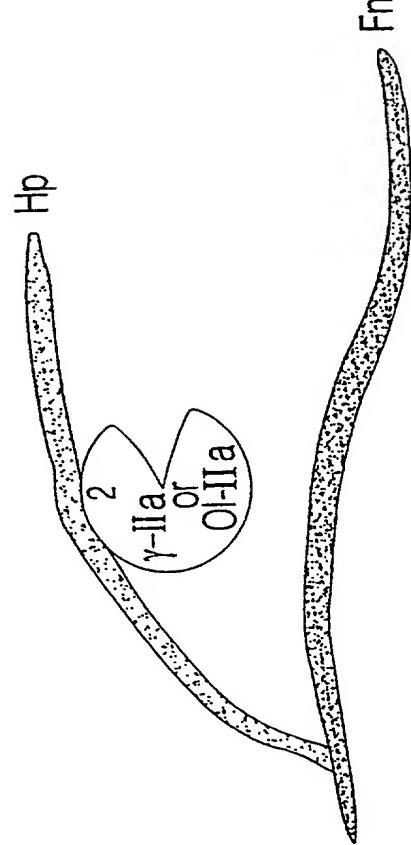


Fig. 7



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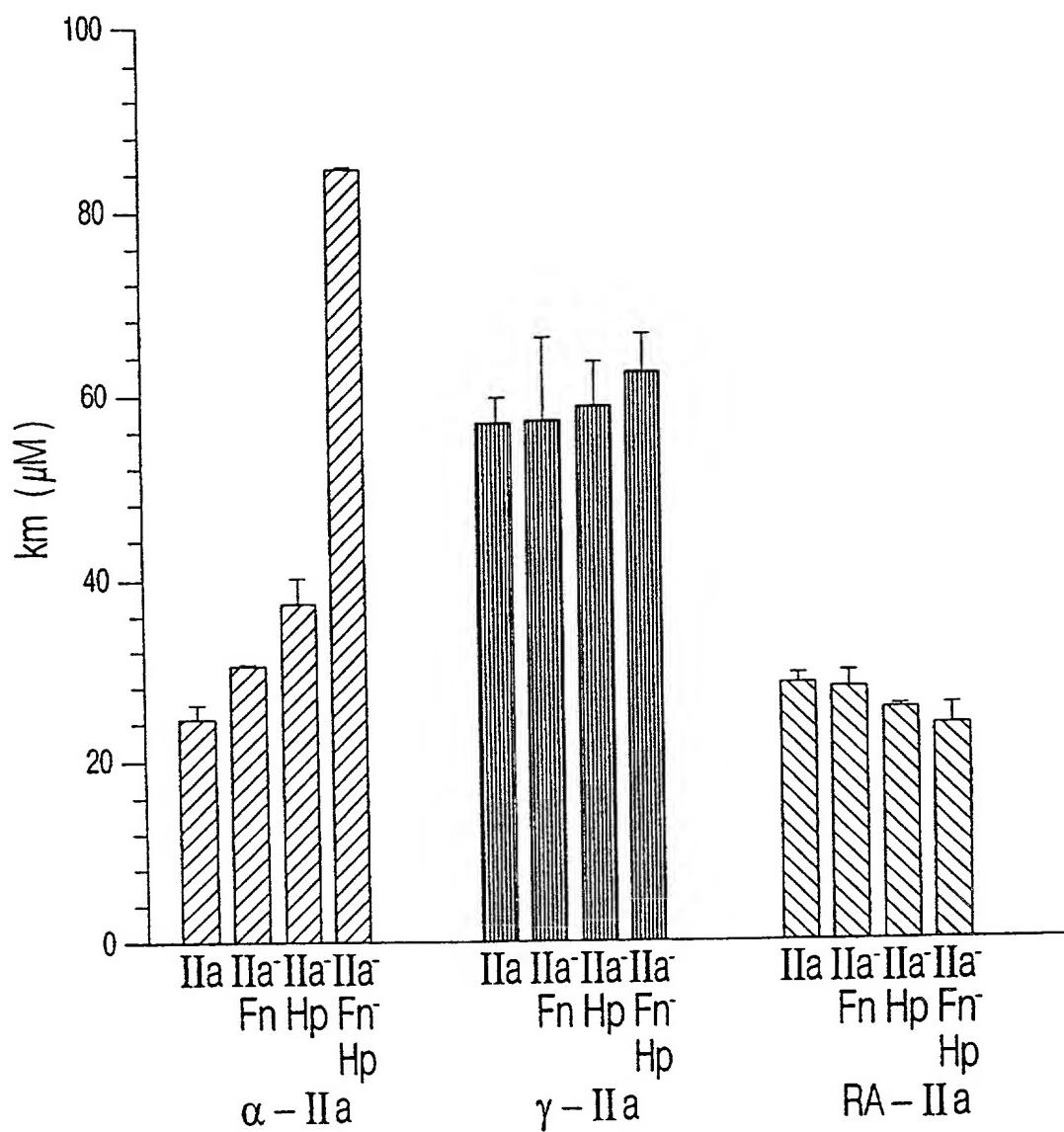


Fig. 8

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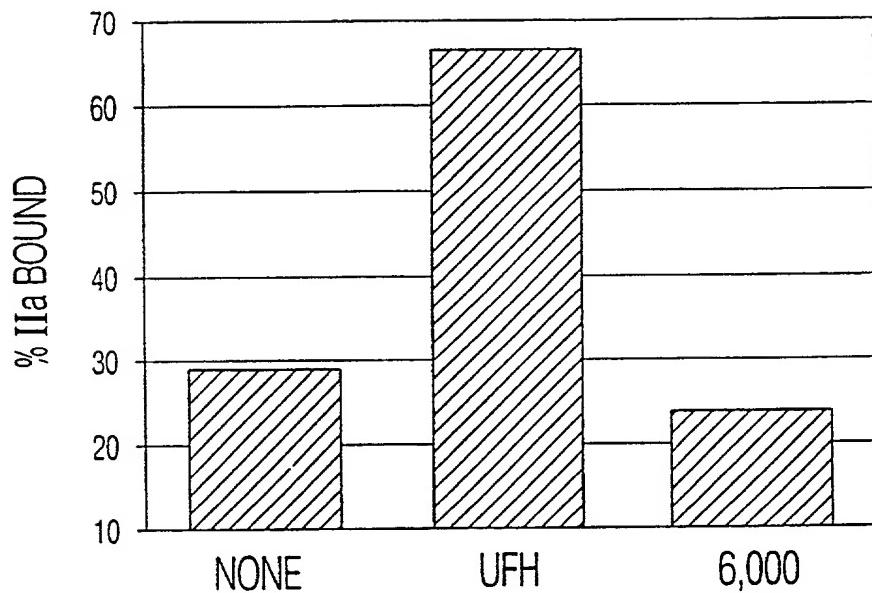


Fig. 9

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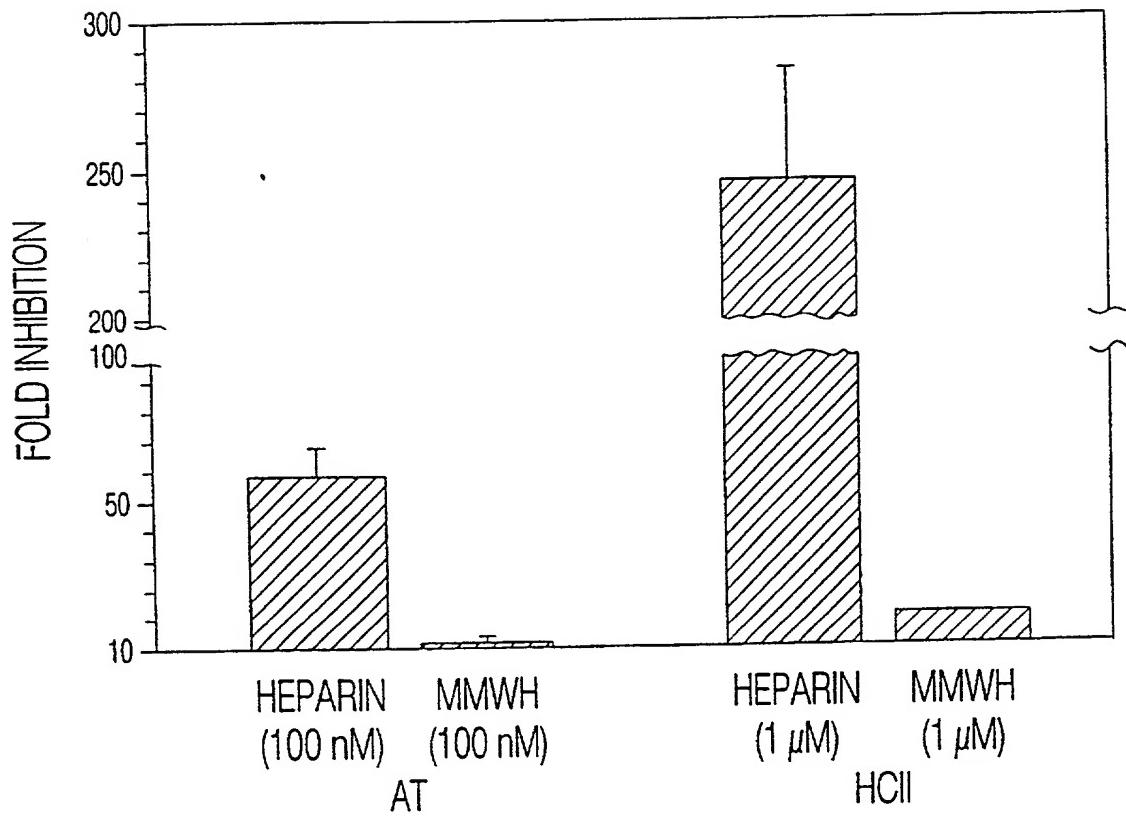
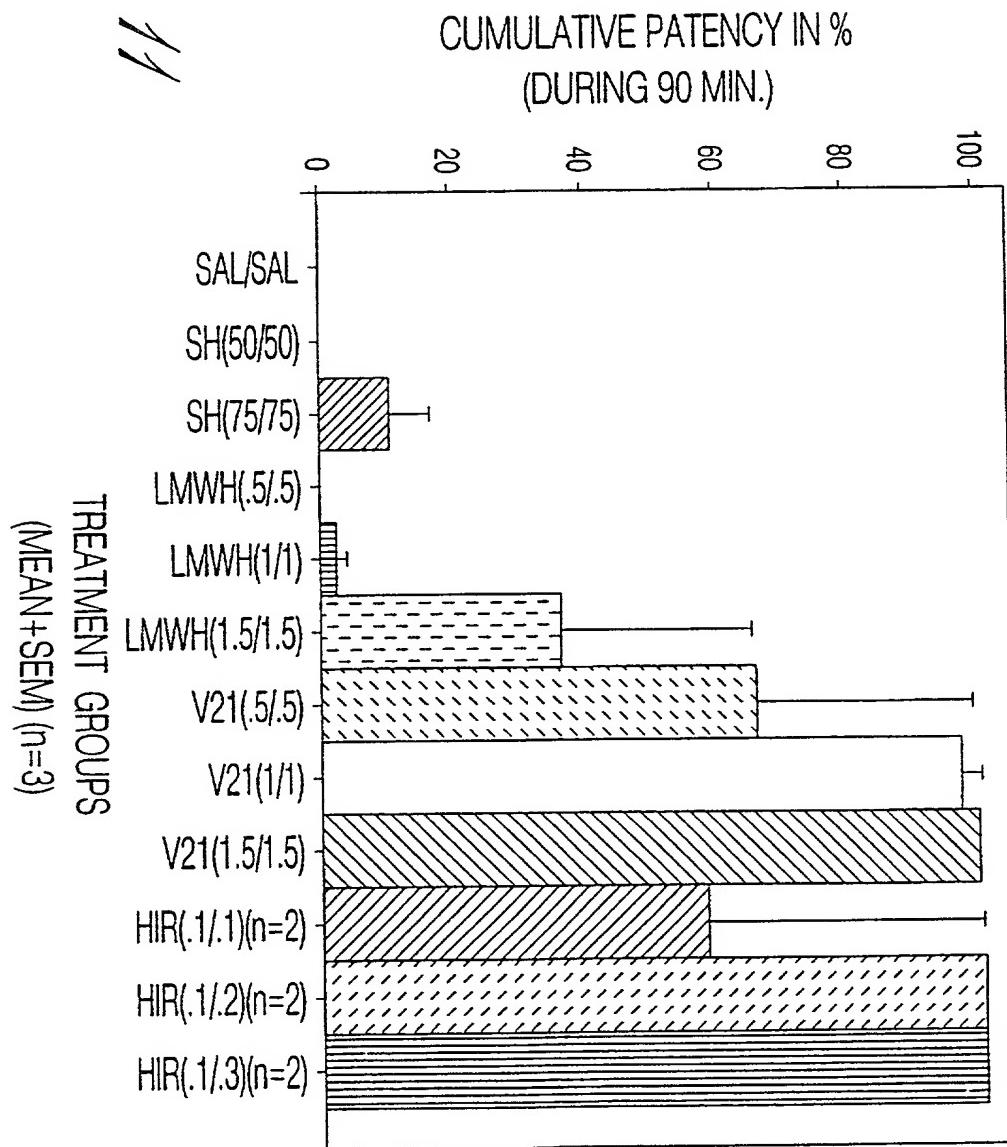


Fig. 10

Fig. 11



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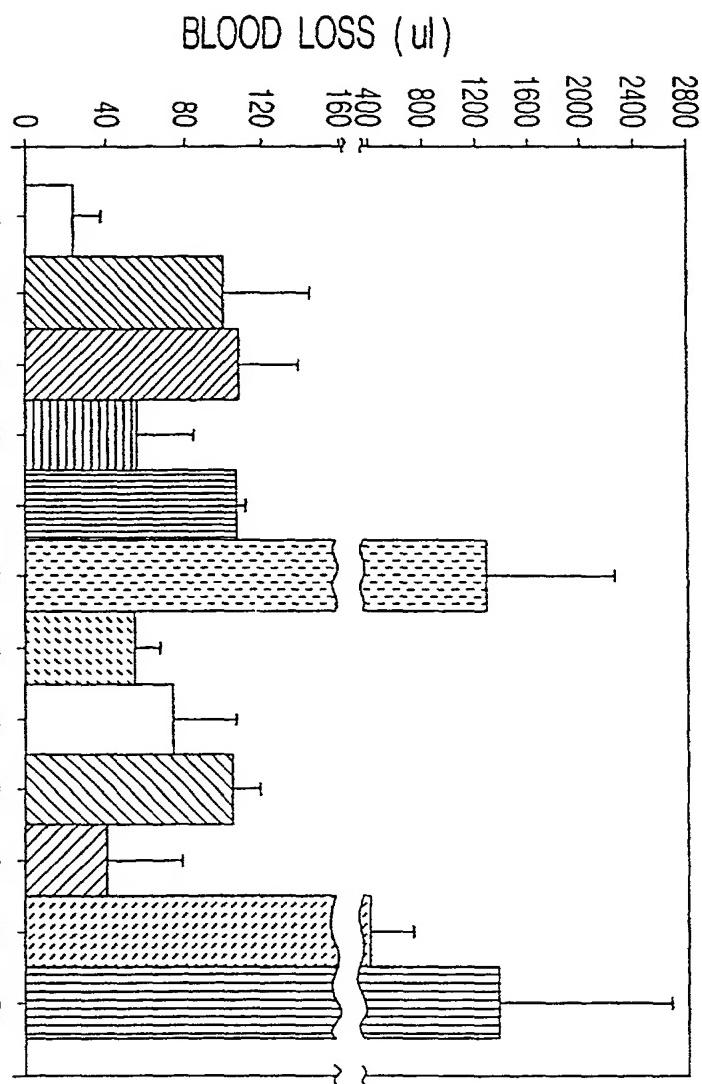
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Fig. 12

TREATMENT GROUPS
(MEAN+SEM) (n=3)



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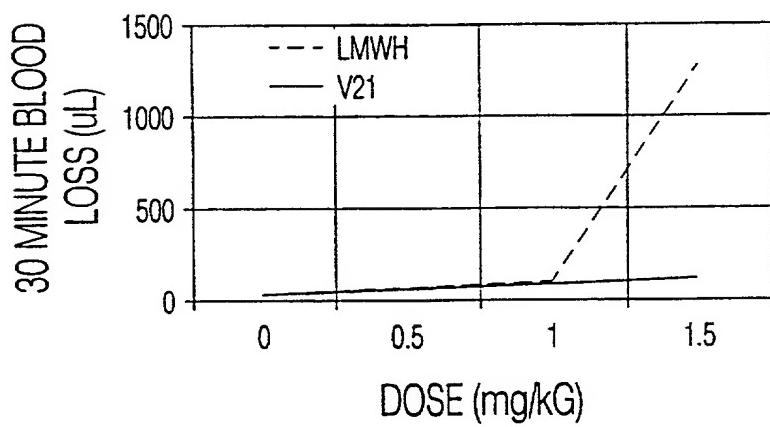
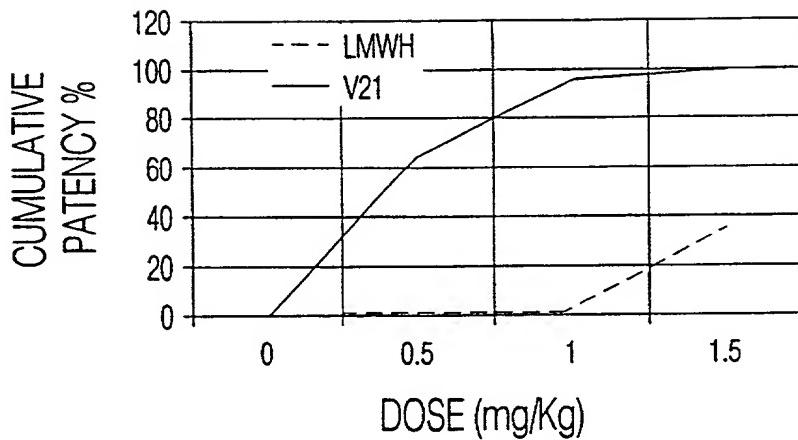


Fig. 13

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	LMWH	V21		
0	21	21	0	0
0.5	24.7	23.1	12.4	1.12
1	24.3	30.7	2.37	10.1
1.5	39.4	52.1	2.98	2.31

Comparative effects of V21 and LMWH on APTT

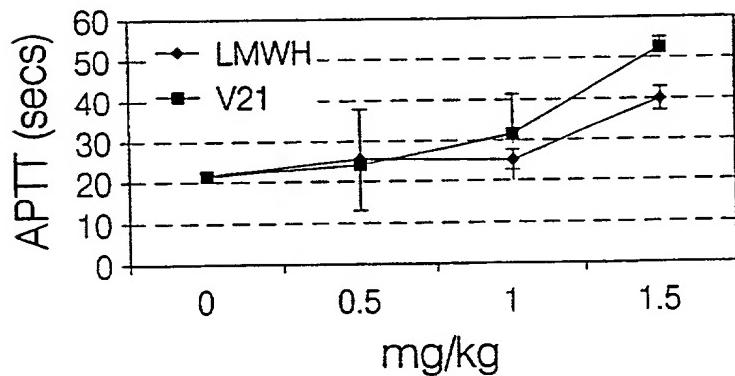


Fig. 14

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LMWH	V21	lmwhse	V21 se	
0	0	0	0	0
0.5	1.1	0.8	0.19	0.32
1	2.8	1.5	0.91	0.32
1.5	2.6	2.8	0.06	0.36

Comparative effects of LMWH and V21 on the anti-Xa level

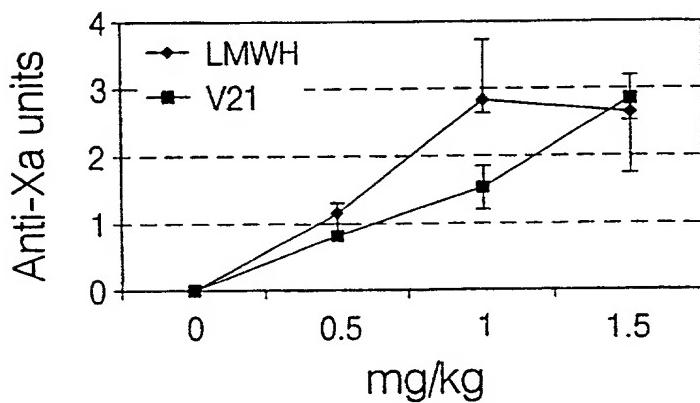
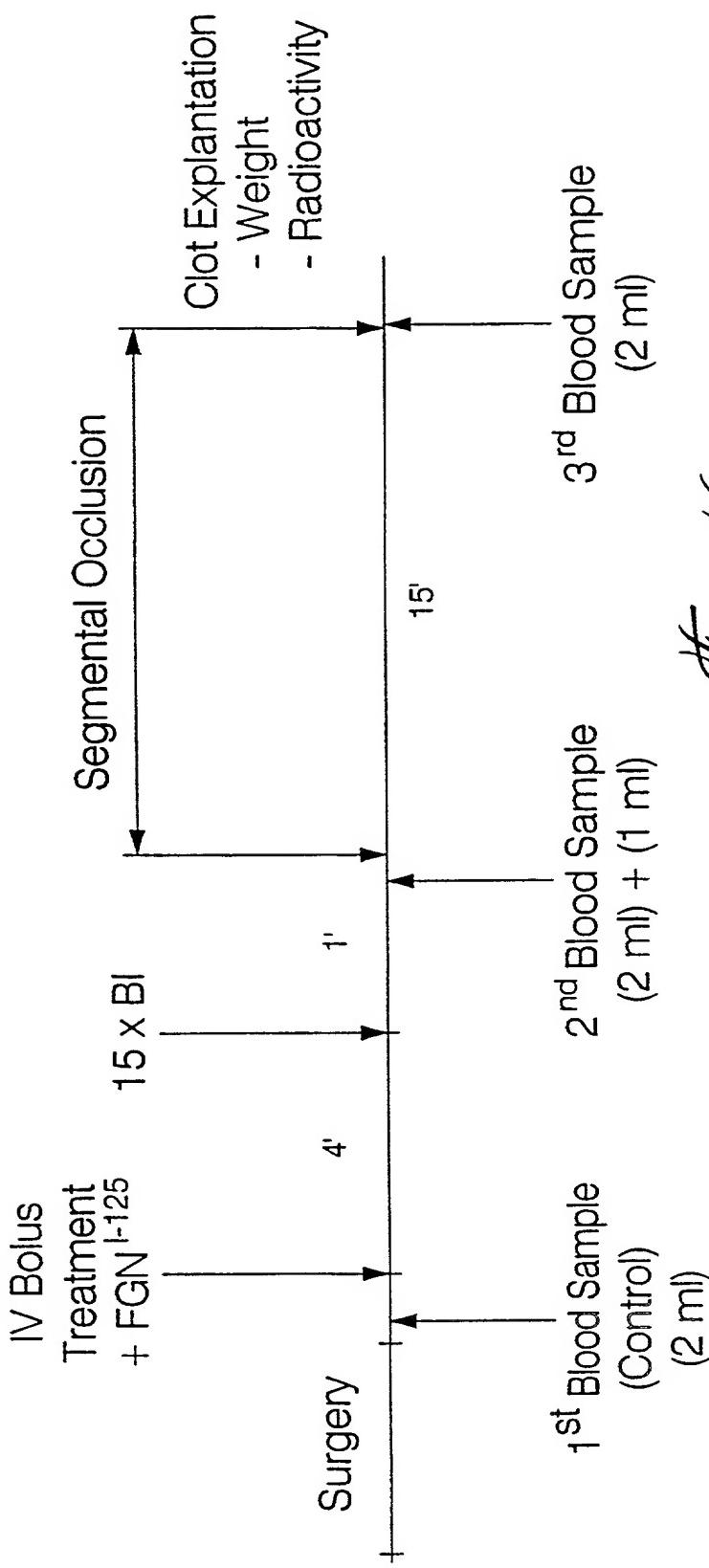


Fig. 15

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Schematic Diagram of the Procedure

*Fig. 16*

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Modified Wessler Model (V-21) Clot Weight (%)

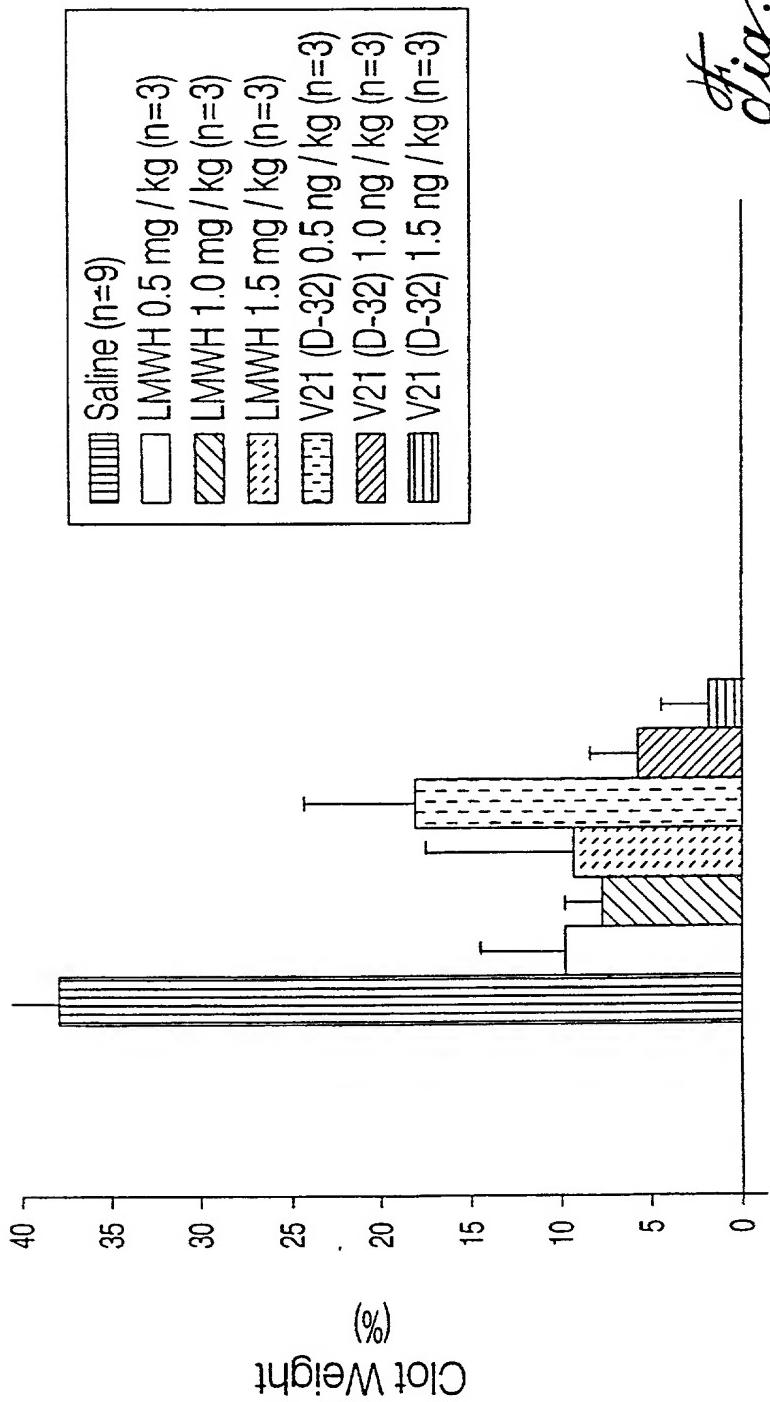


fig. 17

Treatment Groups

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LMWH V21

0	37.8	37.8	3.7	3.7
0.5	9.6	18	4.7	6.1
1	7.5	5.6	2.1	2.6
1.5	9.1	1.7	8.2	0.6

Comparison of LMWH and V21:Prophylaxis model

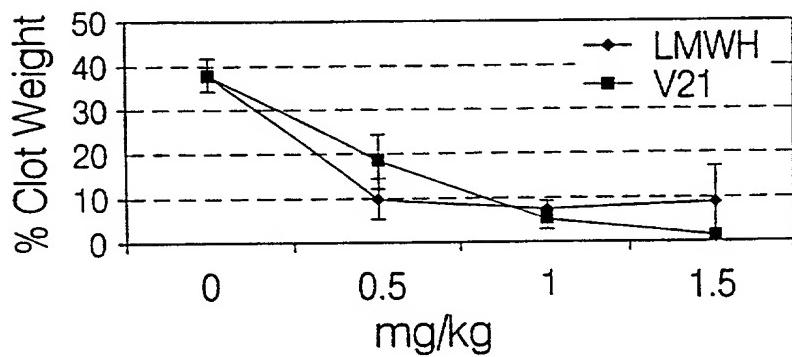


Fig. 18

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Comparison of LMWH and V21:Prophylaxis model

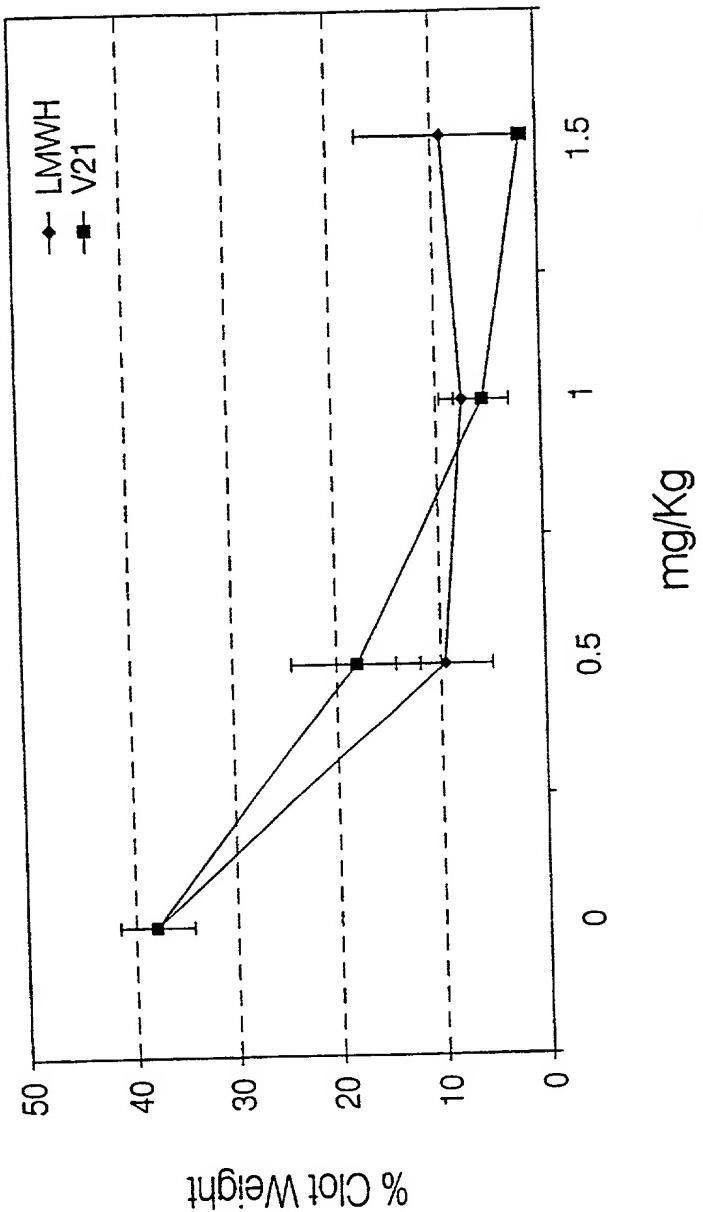


Fig. 19

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Modified Wessler Model (V-21) Clot Radioactivity (%)

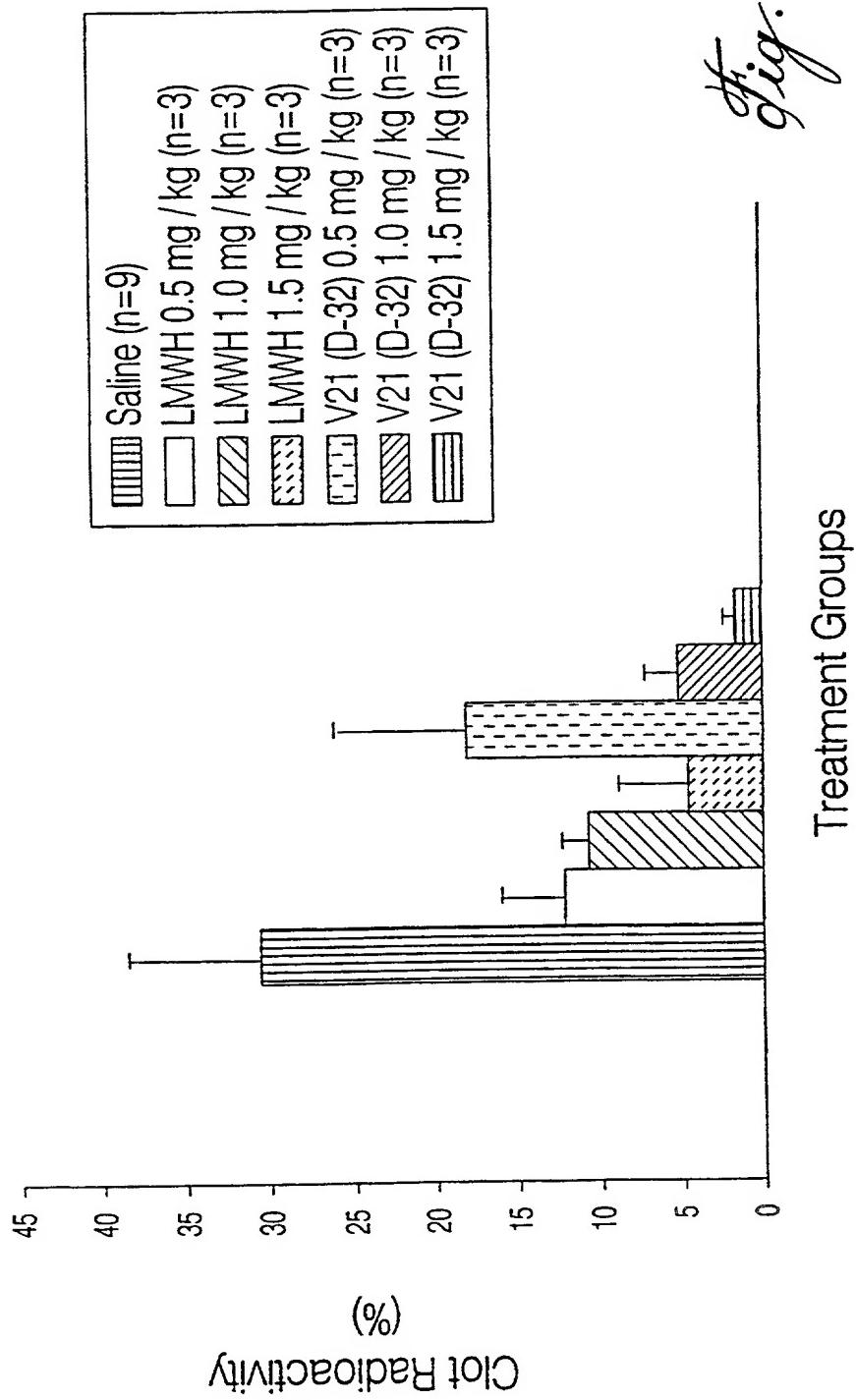


Fig. 20

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LMWH	V21	se	se
0	30	30	8
0.5	12	18	3.8
1	11	5	1.7
1.5	4.5	1.6	4.2

Comparison of LMWH and V21: Prophylaxis Model

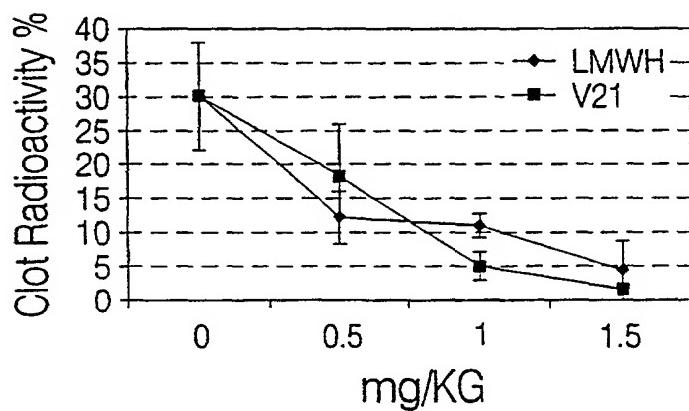


Fig. 21

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Comparison of LMWH and V21:Prophyaxis Model

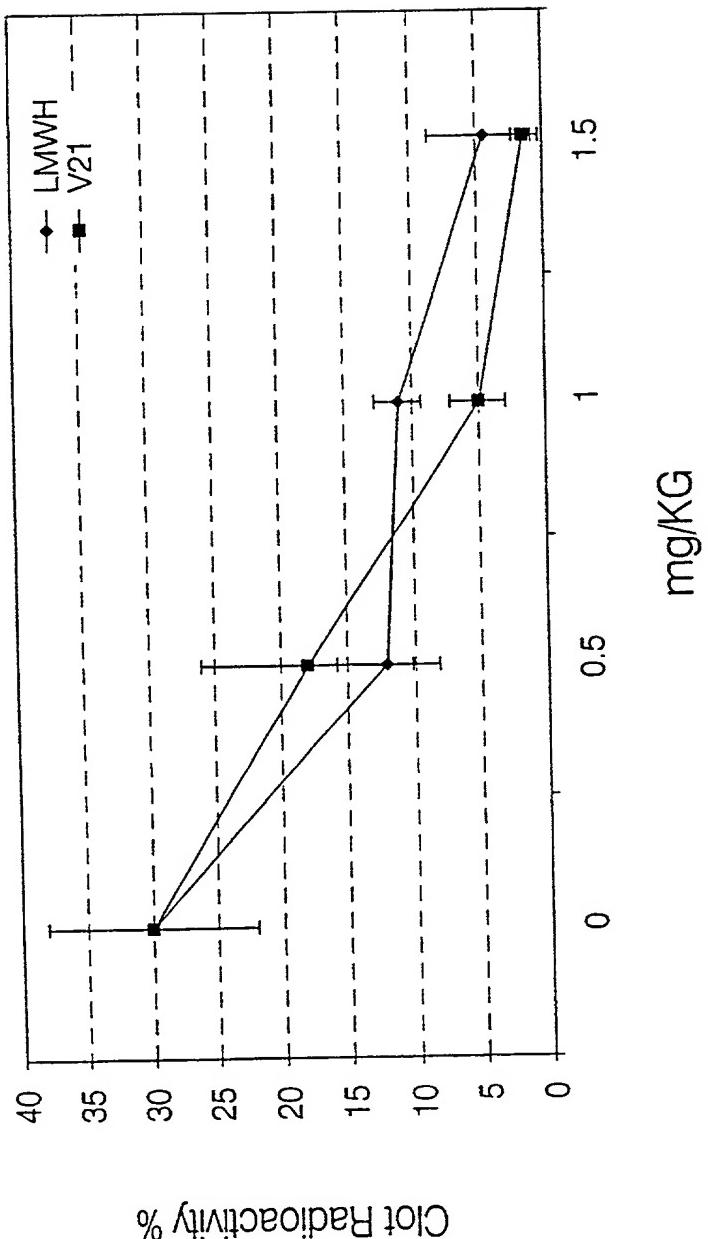
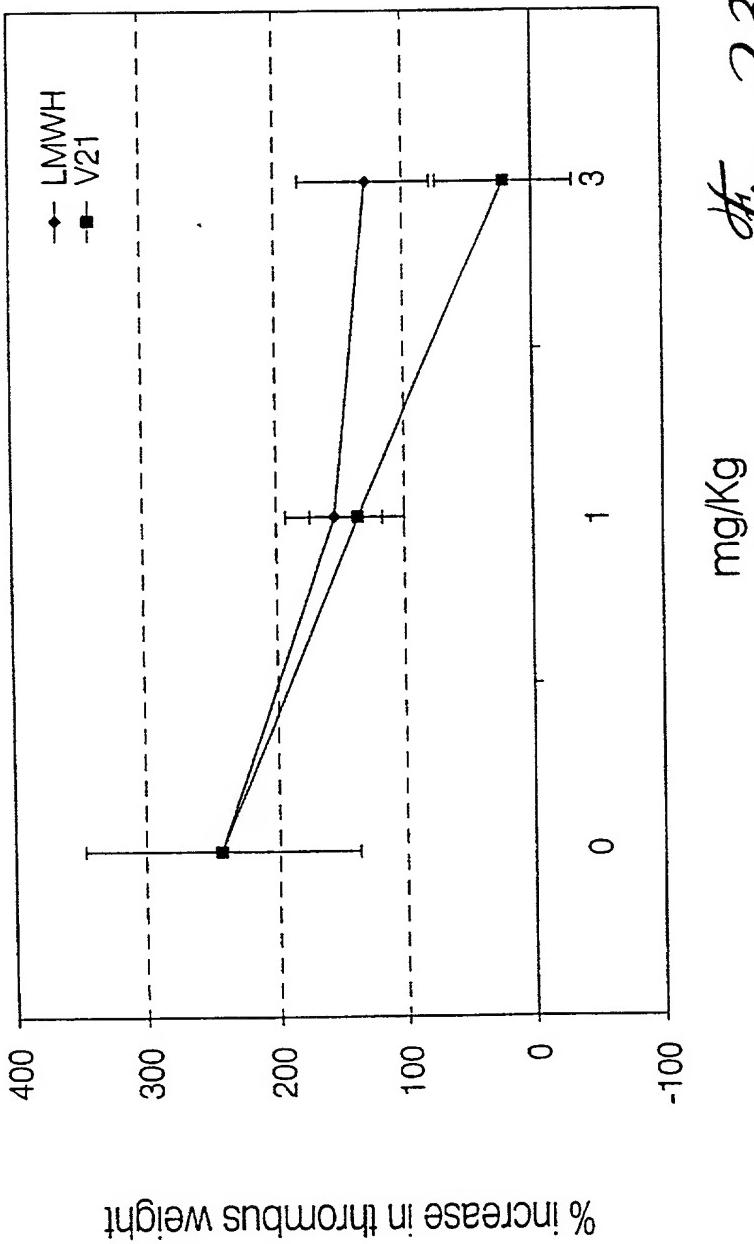


Fig. 22

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Comparison of LMWH and V21 in Treatment Model

Fig. 23
mg/kg

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LMWH V21

0	242	242	104	104
1	155	137	37	30
3	129	21	52	31

Comparison of LMWH and V21 in Treatment Model

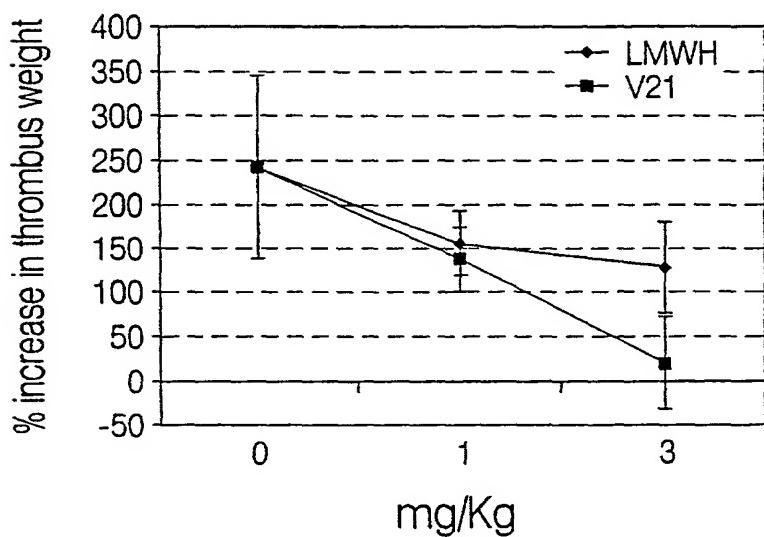


Fig. 24

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LMWH and V21 on Thrombus Accretion

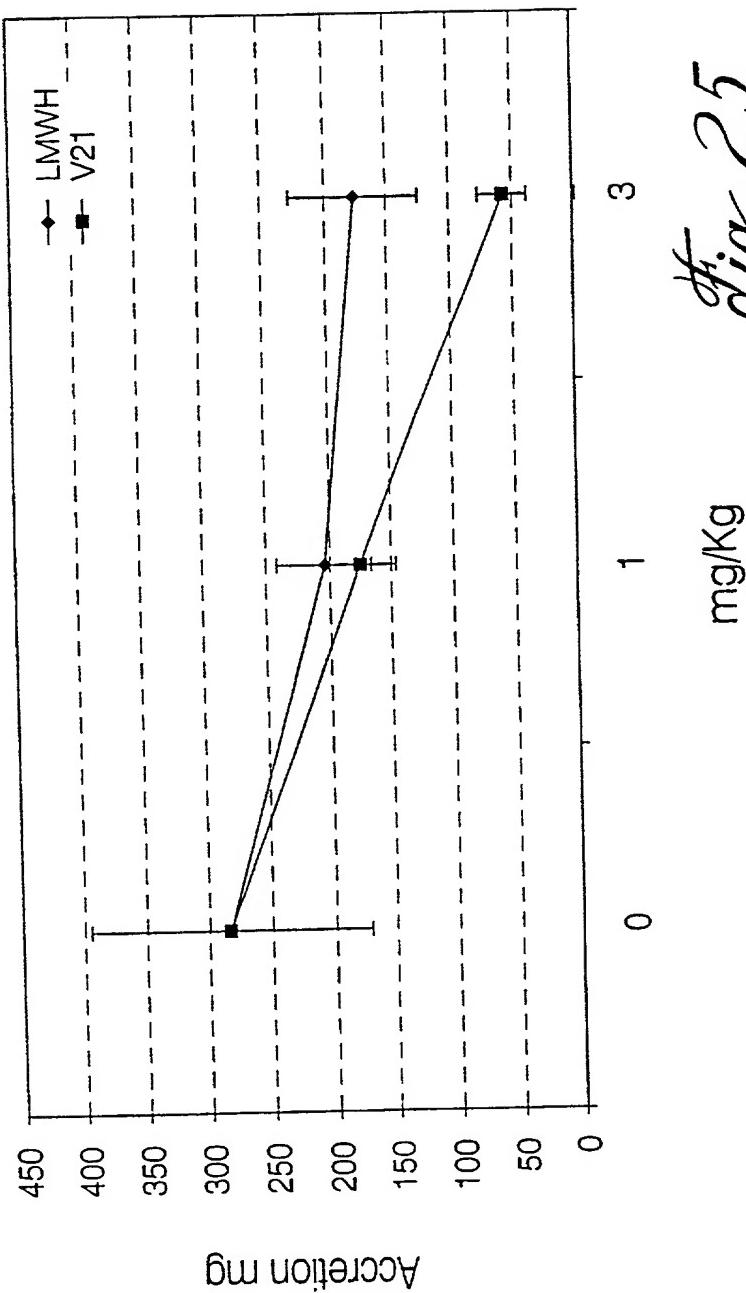


fig. 25

mg/Kg

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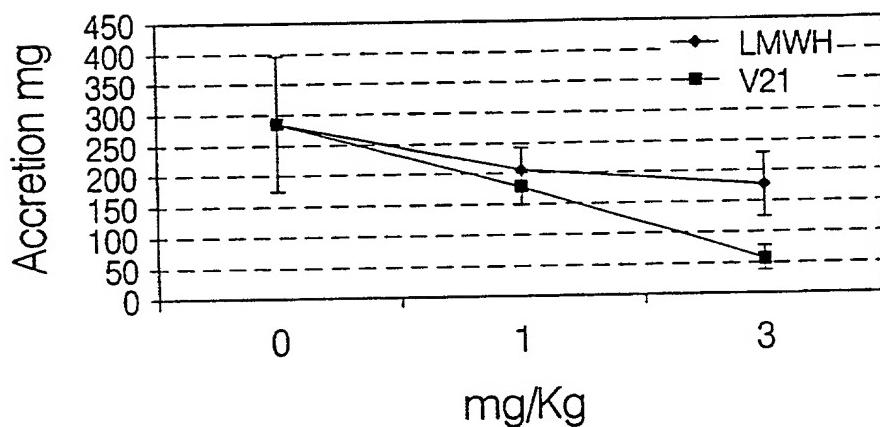
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LMWH V21

0	282	282	111	111
1	202	174	37	29
3	174	57	51	20

LMWH and V21 on Thrombus Accretion

*Fig. 26*

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V21 Treatment of DVT in Chronic Rabbit Model Clot Accretion

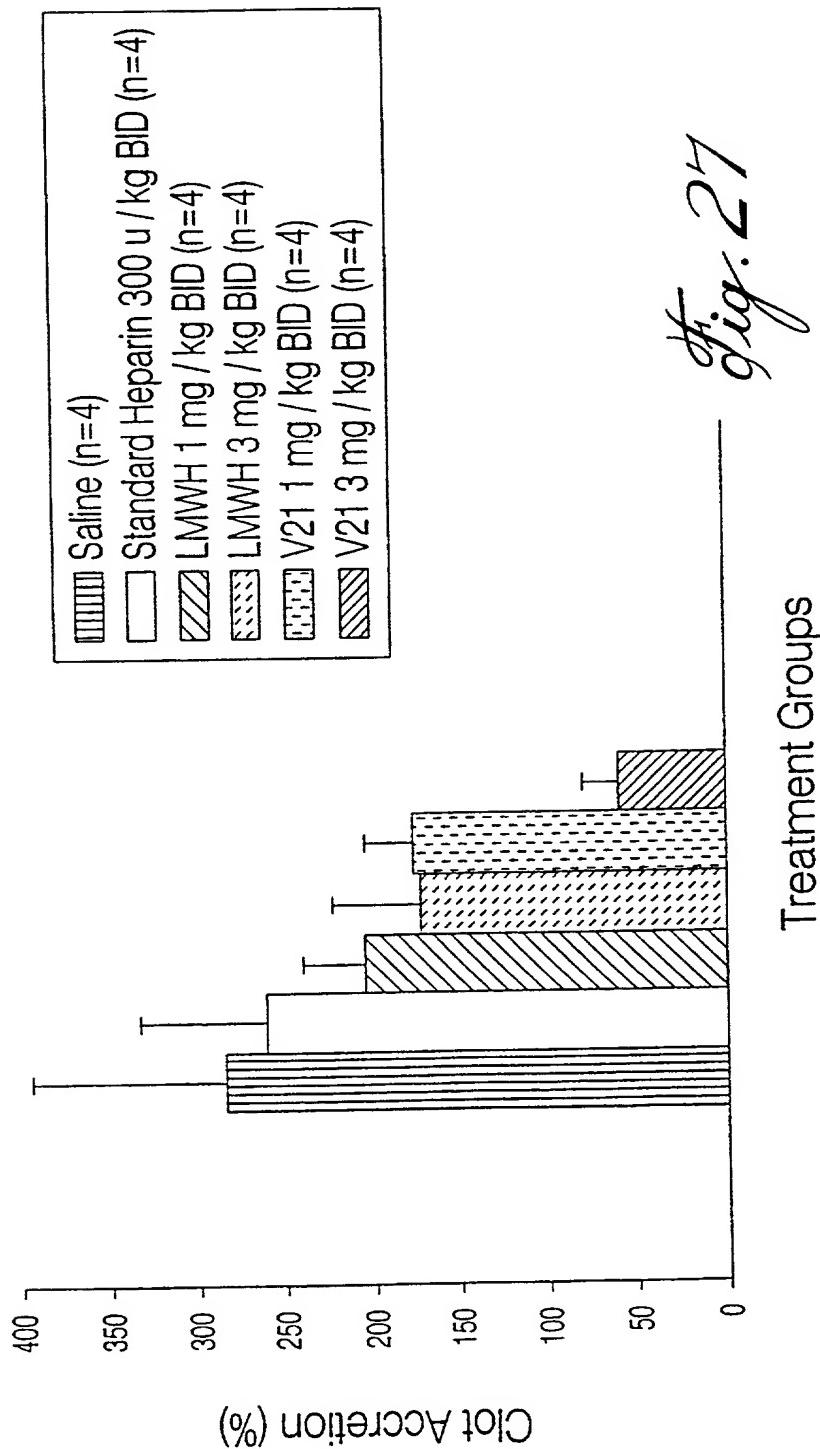


fig. 27

Treatment Groups

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V21 Treatment of DVT in Chronic Rabbit Model % Change in Clot Weight

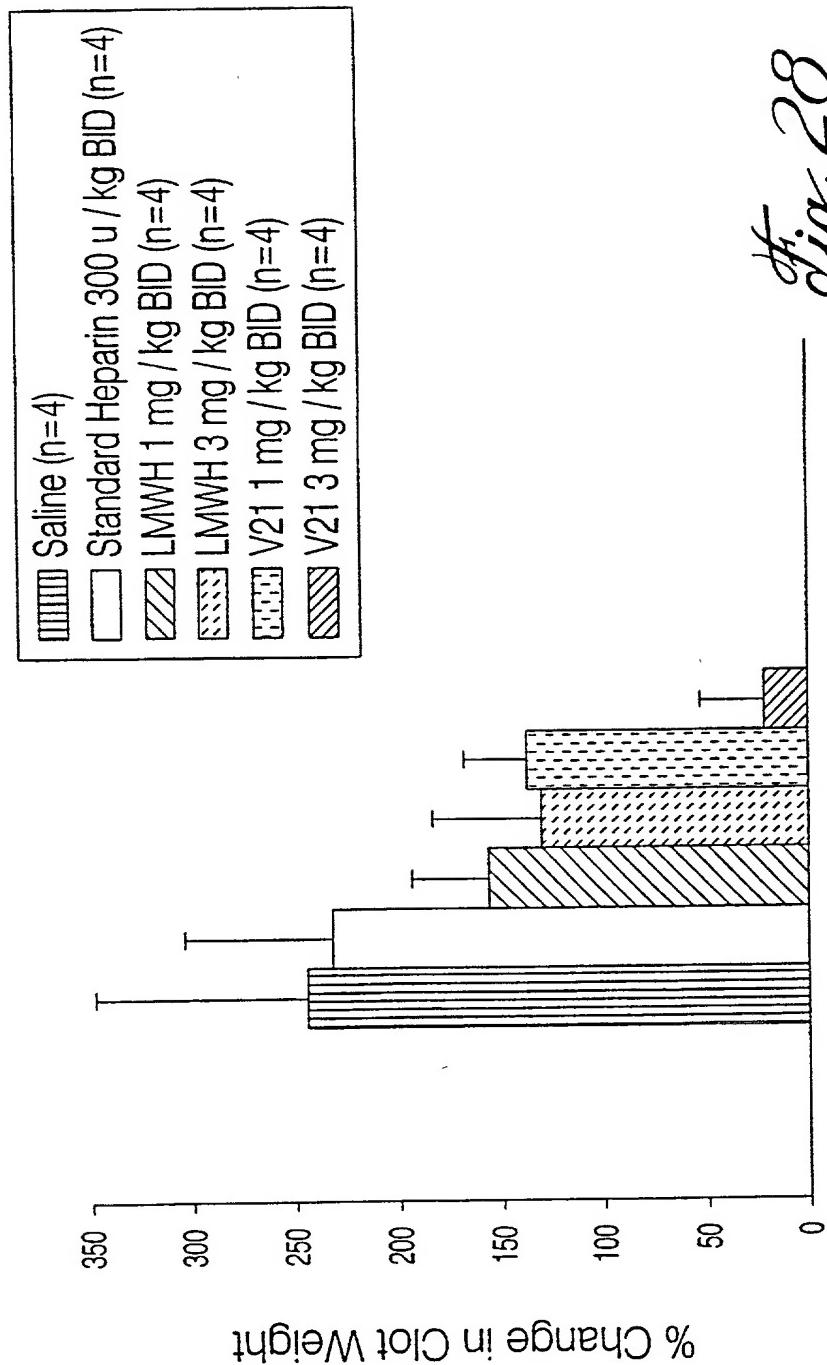


fig. 28

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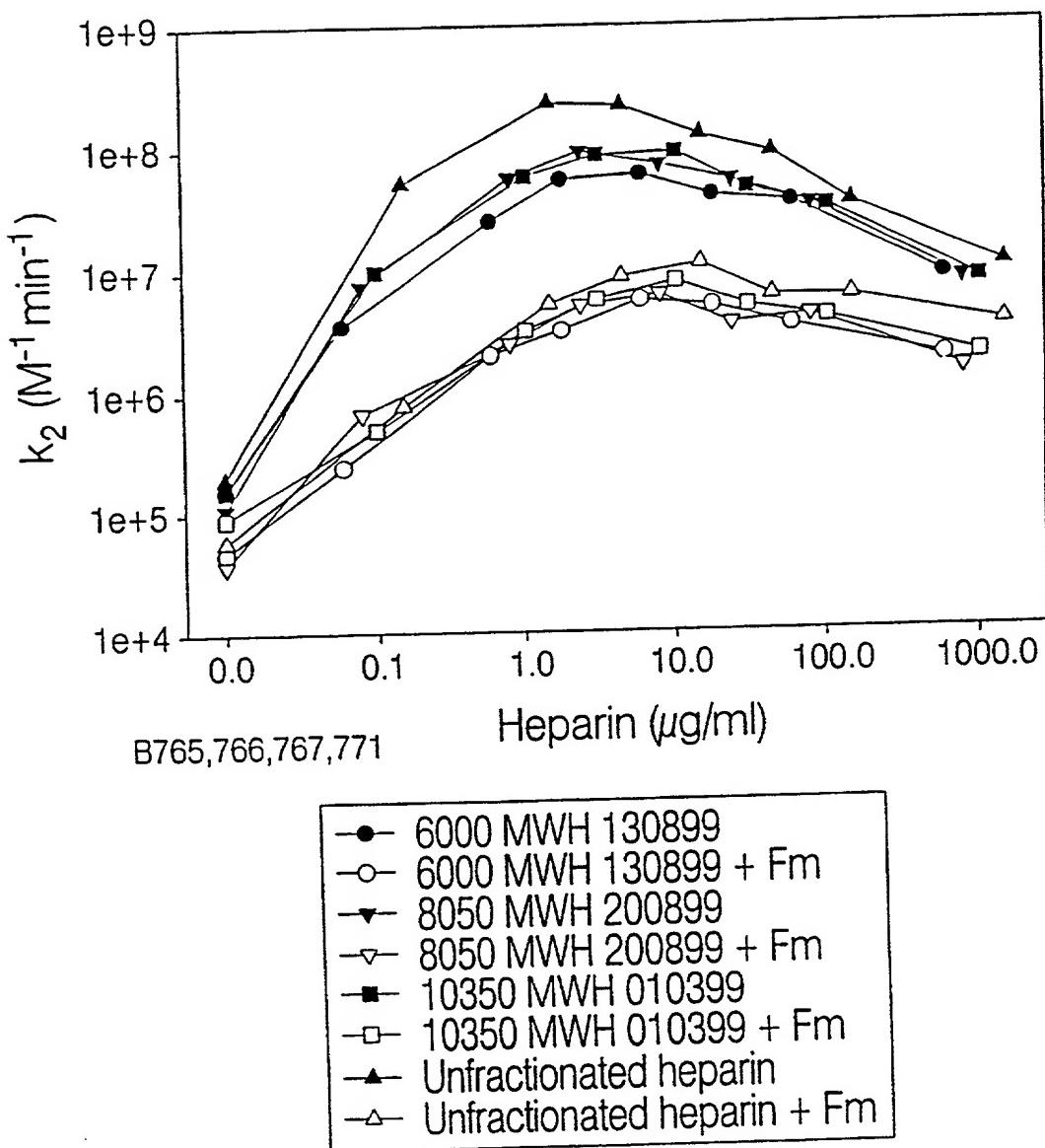
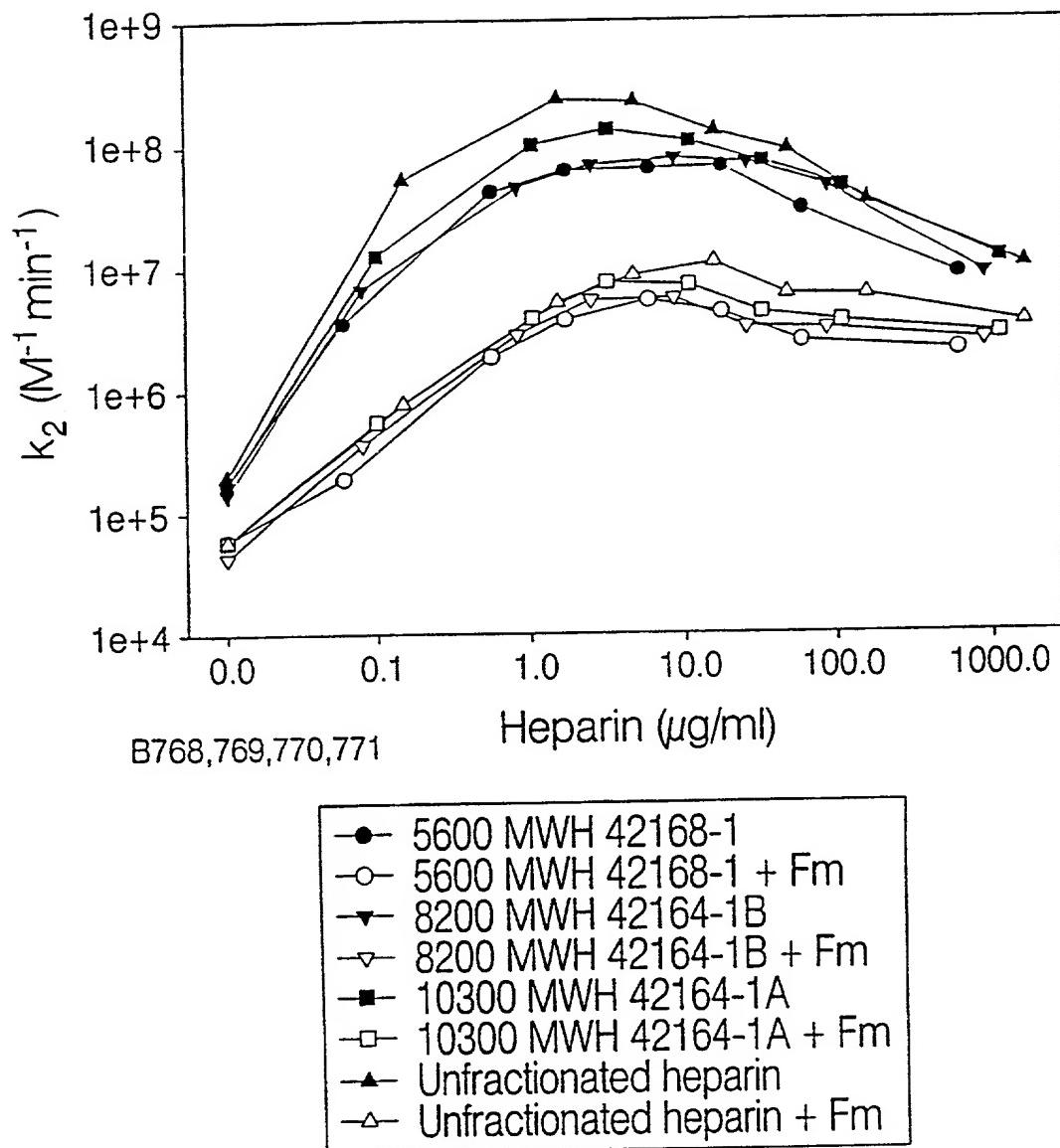
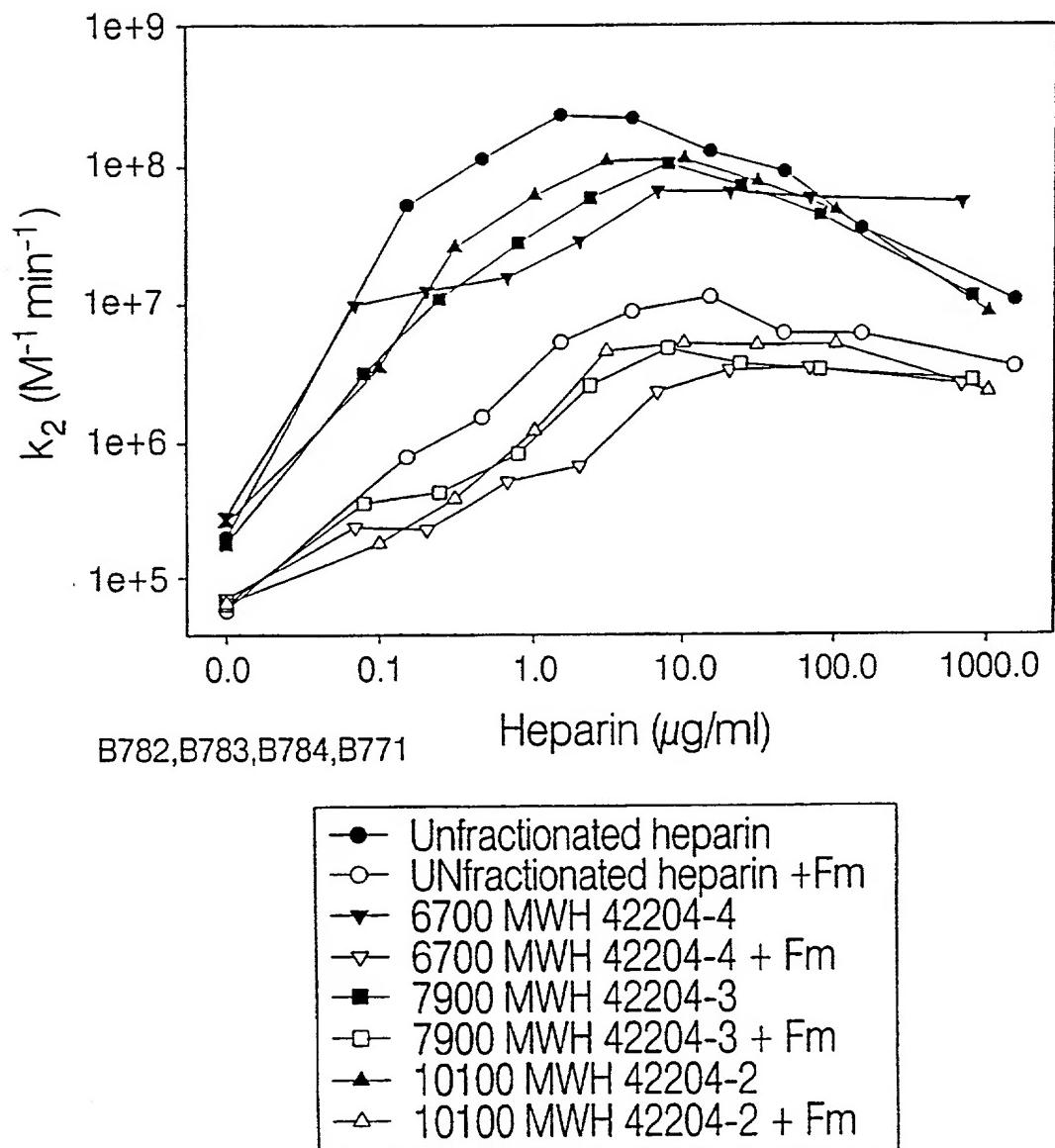


Fig. 29

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*Fig. 30*

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*Fig. 31*

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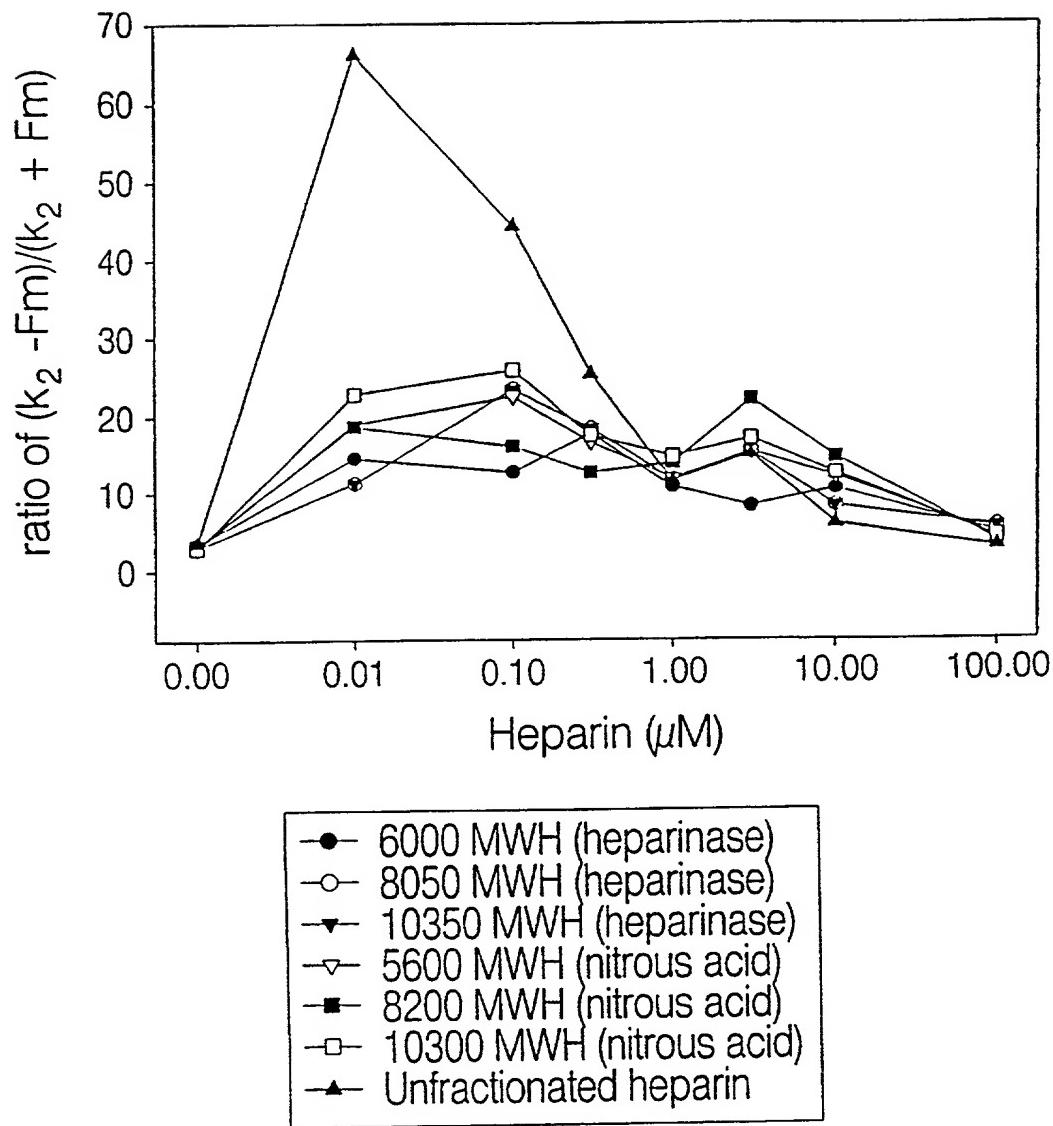


Fig. 32

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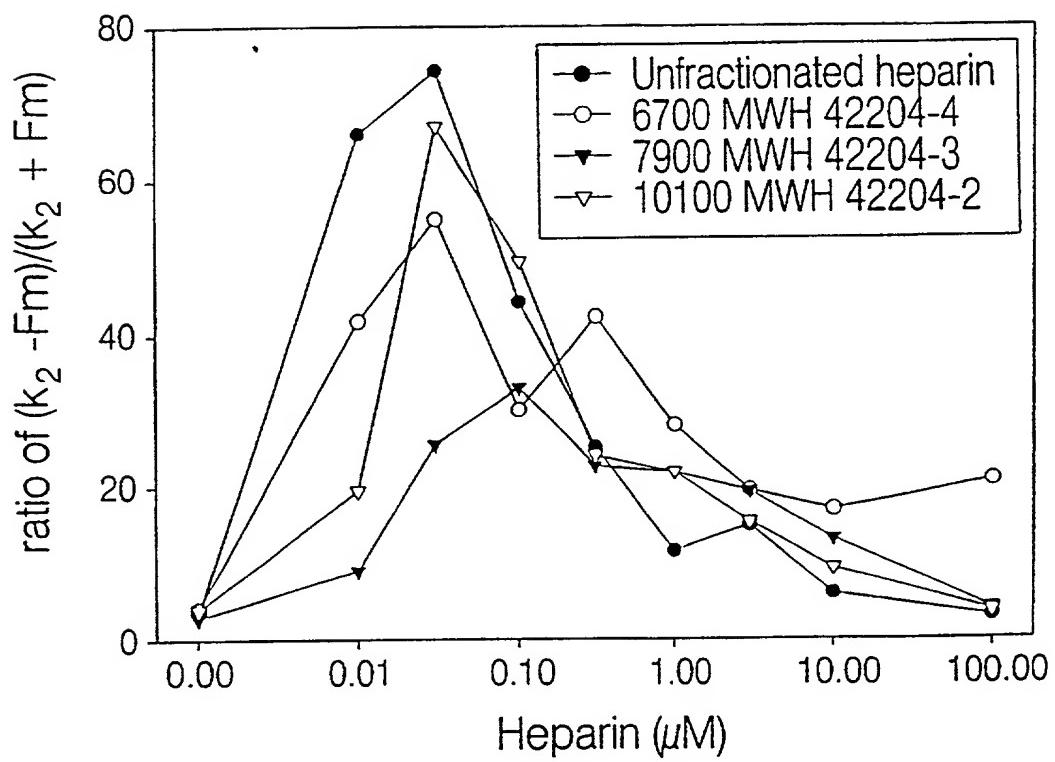


Fig. 33

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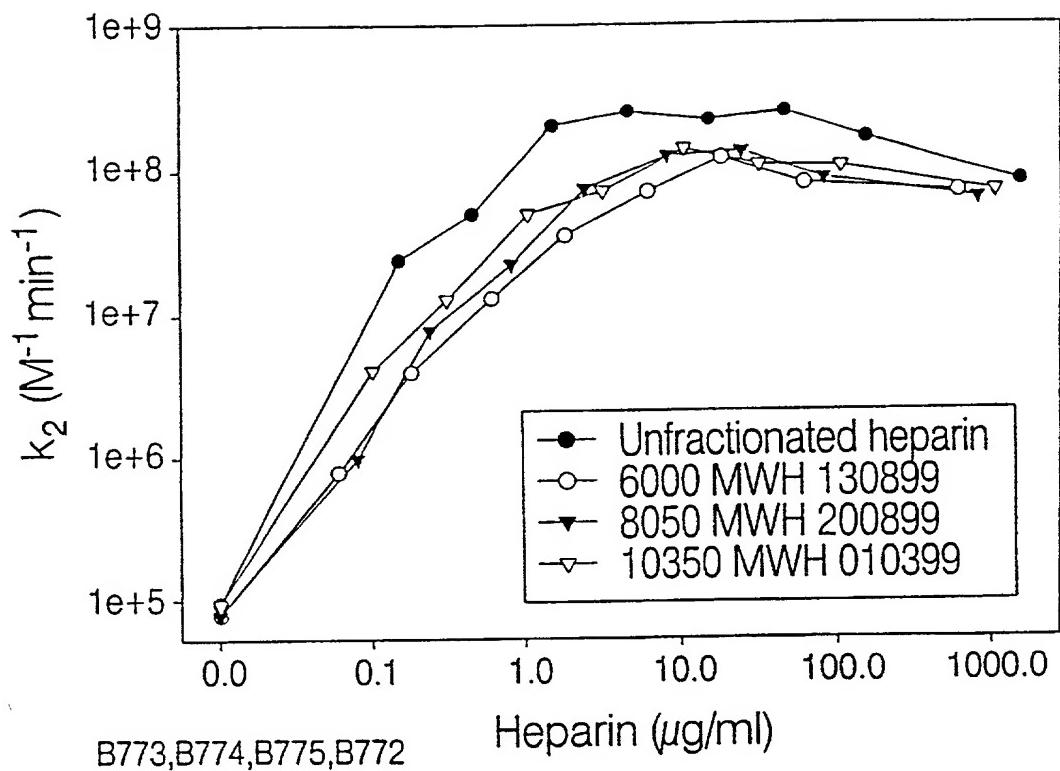
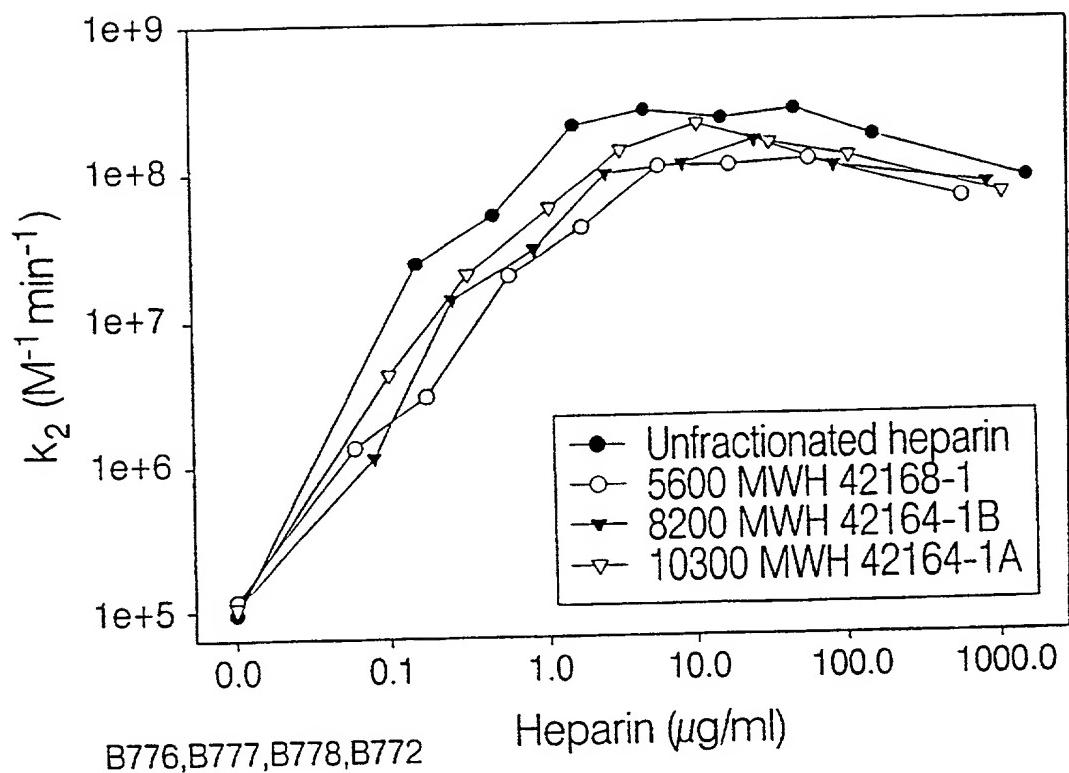


Fig. 34

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B776,B777,B778,B772

Fig. 35

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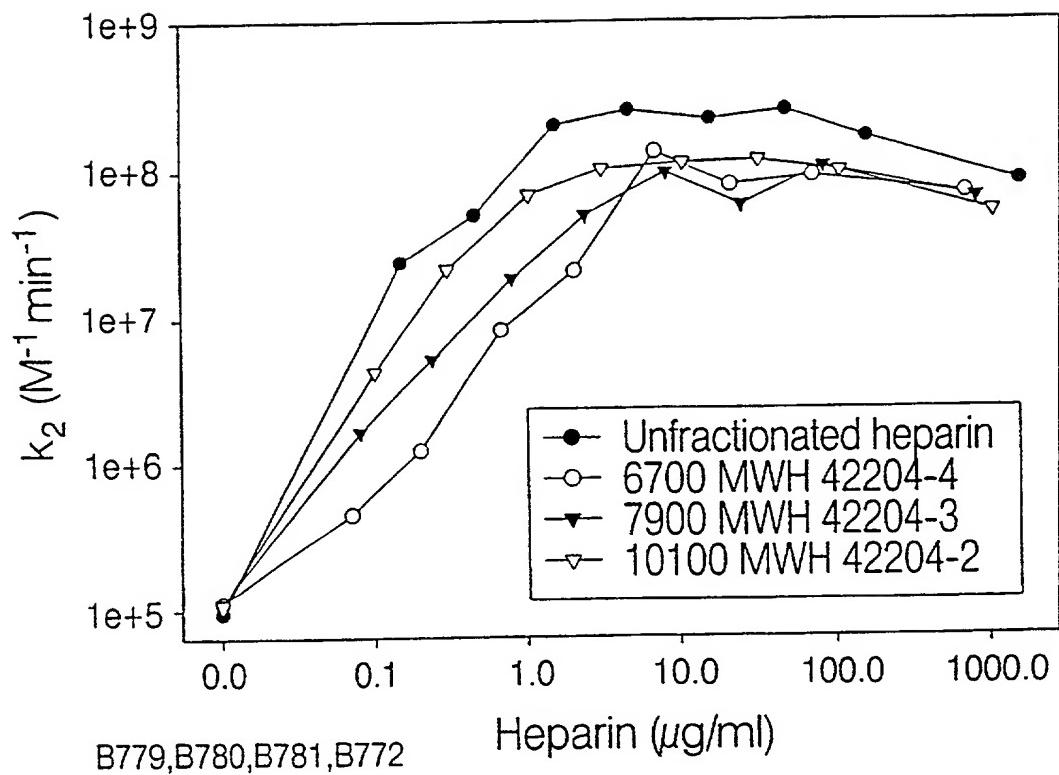


Fig. 36

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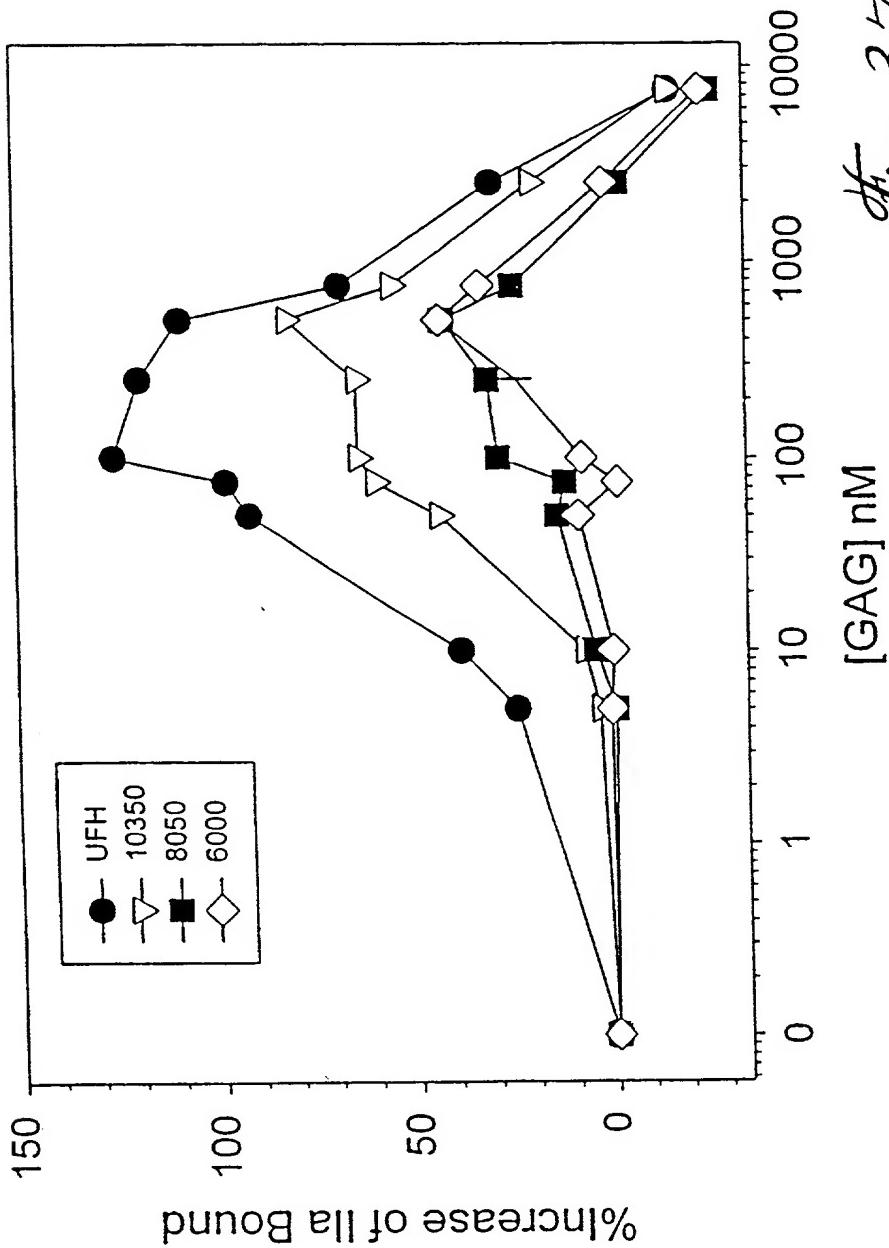
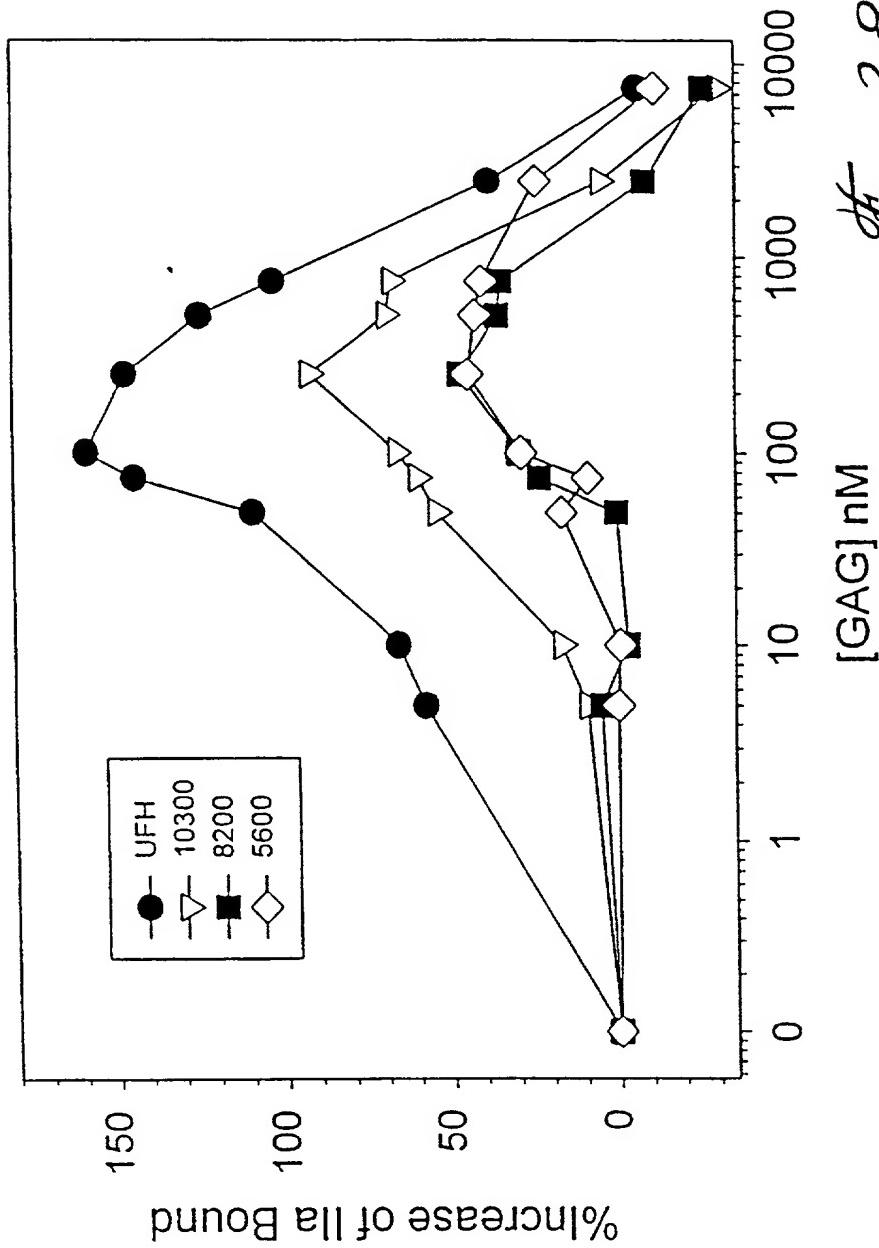


Fig. 37

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*Fig. 38*

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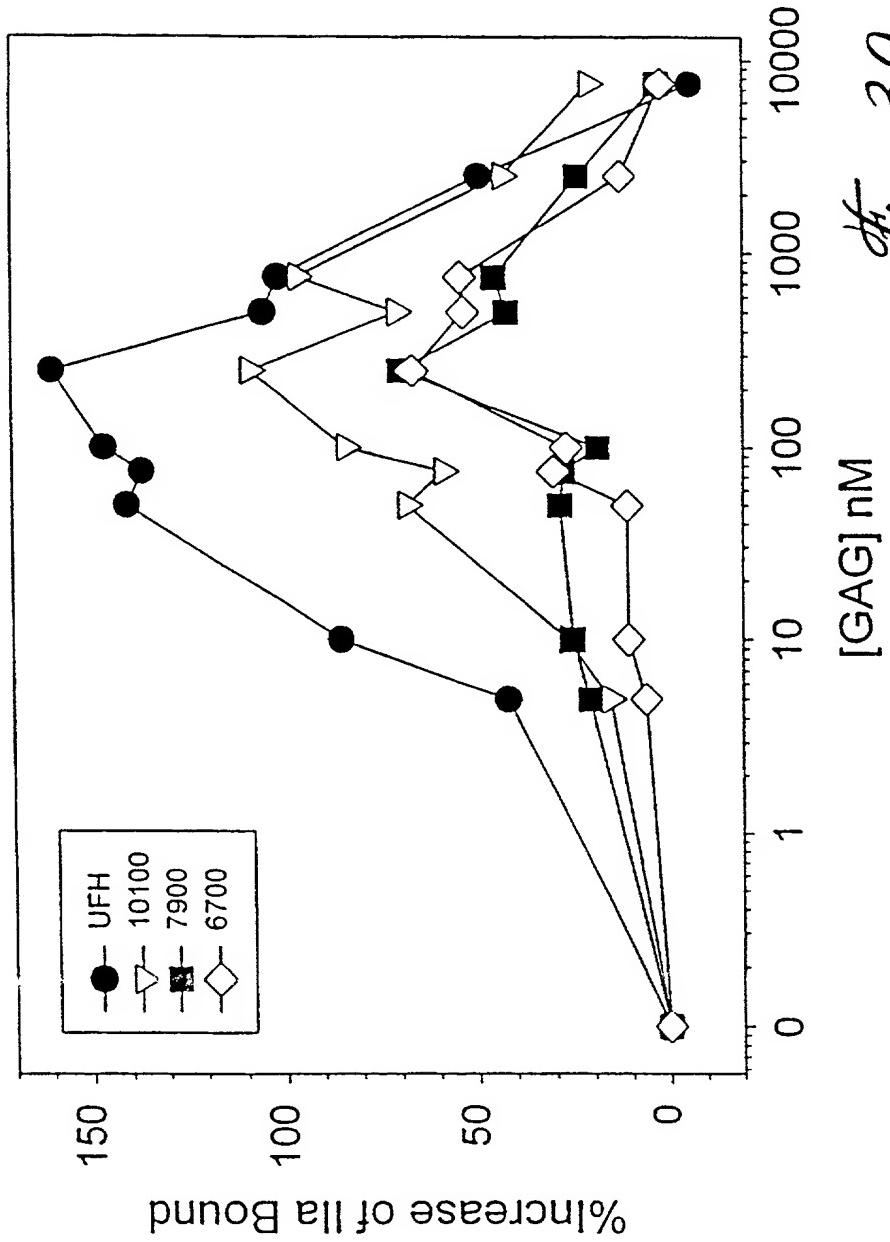
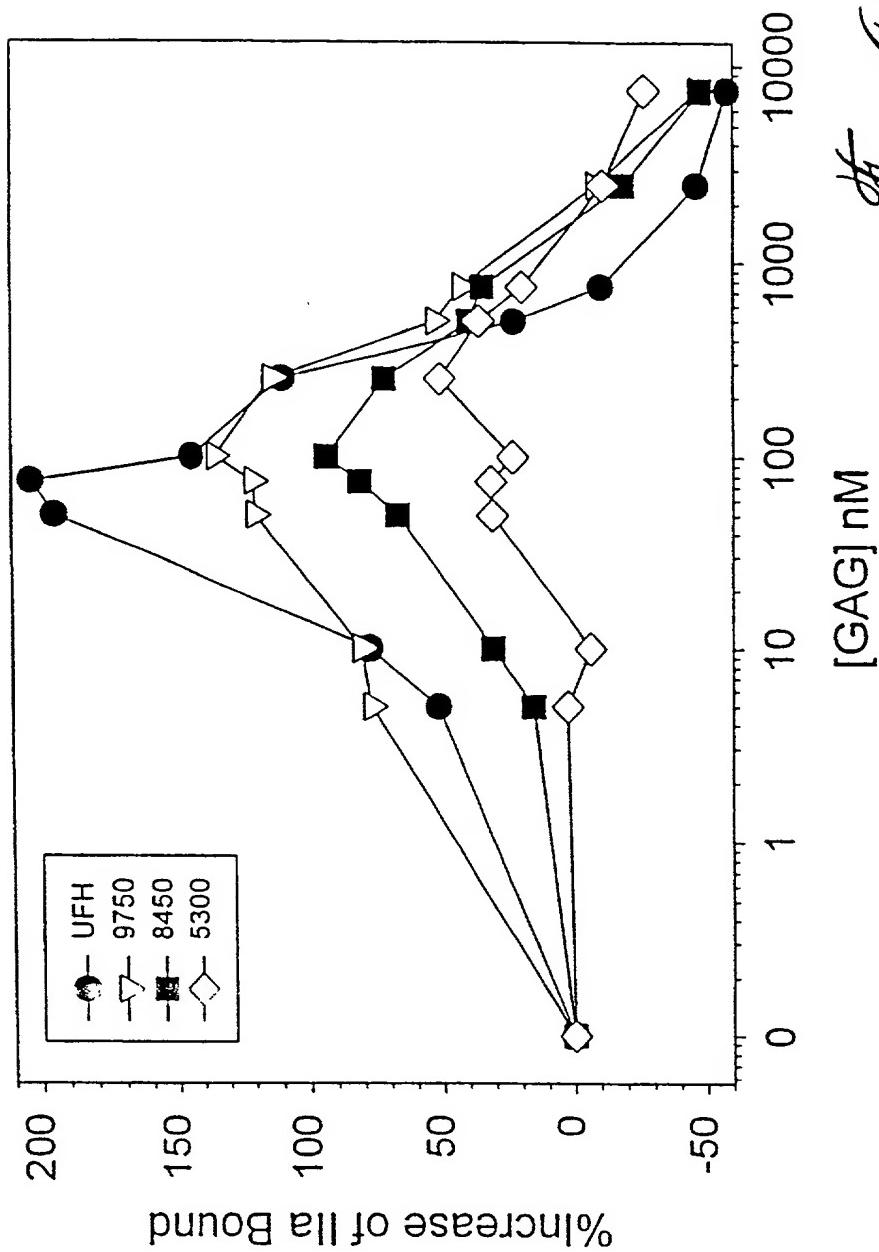


fig. 39

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*Fig. 40*

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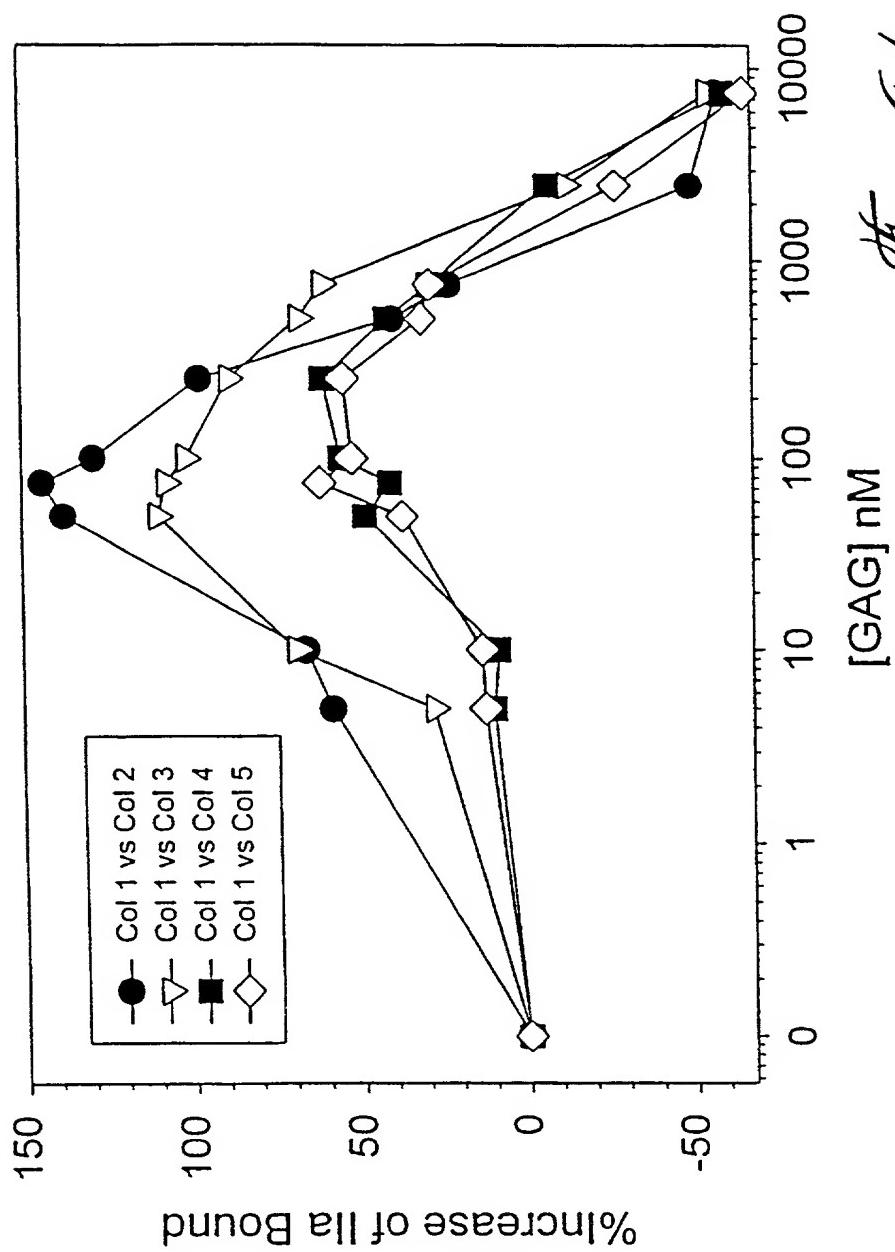


Fig. 41

DECLARATION FOR PATENT APPLICATION

Docket Number GDV-001.01 #5

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

HEPARIN COMPOSITIONS THAT INHIBIT CLOT ASSOCIATED COAGULATION FACTORS

the specification of which (check one)

is attached hereto.

was filed on Date June 29, 2000 as United States Application Number or PCT International Application Number PCT/CA00/00774, and was amended on Date _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information, which is material to patentability as defined in Title 37, Code of Federal Regulation, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Priority Claimed

Yes No

Yes No

(Number)	(Country)	(Day/Month/Year Filed)
----------	-----------	------------------------

(Number)	(Country)	(Day/Month/Year Filed)
----------	-----------	------------------------

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States Provisional application(s) listed below.

60/ <u>141,865</u>	<u>30 June 1999</u>
(Application Number)	(Filing Date)

60/ <u>154,744</u>	<u>17 September 1999</u>
(Application Number)	(Filing Date)

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

<u> </u>	<u> </u>	(Status: patented, pending, abandoned)
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<u> </u>	<u> </u>	(Status: patented, pending, abandoned)
---------------	---------------	--

I hereby appoint Beth E. Arnold, Reg. No. 35,430; Isabelle Clauss, Reg. No. 47,326; Stephen B. Deutsch, Reg. No. 46,663; William D. DeVaul Reg. No. 42,483; Daniel P. Gaudet, Reg. No. 48,584; Robert W. Gauthier, Reg. No. 35,153; Jason Gish, Reg. No. 42,581; Dana Gordon, Reg. No. 44,719; Robert A. Greenberg, Reg. No. 44,133; Jennifer K. Holmes, Reg. No. 46,778; David Hyams, Reg. No. P-50947; Scott E. Kamholz, Reg. No. 48,543; Theresa Kavanaugh, Reg. No. P-50356; David A. Lane, Reg. No. 39,261; W. Hugo Liepmann, Reg. No. 20,407; Jim Olesen, Reg. No. 46,967; Kevin A. Oliver, Reg. No. 42,049; Chinh H. Pham, Reg. No. 39,329; Jeffrey M. Sears, Reg. No. 48,440; Philip C. Swain, Reg. No. 32,376; Kingsley L. Taft, Reg. No. 43,946; John Quisel, Reg. No. 47,874; Anita Varma, Reg. No. 43,221; Sharon Webb, Reg. No. 47,172; John L. Welch, Reg. No. 28,129 and Jennifer Zarutskie, Reg. No. P-50558 as attorneys/agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

Address all telephone calls to Anita Varma at telephone number (617) 832-1242.

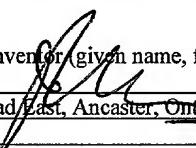
Address all correspondence to: **Customer Id No: 25181**

Patent Group

Foley Hoag & Eliot LLP
One Post Office Square
Boston, MA. 02109-2170

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor (given name, family name): Jeffrey I. Weitz

Inventor's signature: 

Residence: 54 Carluke Road East, Ancaster, Ontario L9G 3L1 CAK

Post Office Address: _____

Date: JAN 2 2002

Citizenship: CANADA

Additional inventors are being named separately numbered sheets attached hereto.

Full name of second joint inventor (given name, family name): Jack Hirsh

Inventor's signature:

Residence: 21 Cottage Avenue, Hamilton, Ontario L8P 4G5

Post Office Address:

CPX

Date: January 8, 2002

Citizenship: CANADA

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